STRUCTURE AND FUNCTION OF SARCOPLASMIC RETICULUM ISOLATED FROM SLOWLY- AND FROM RAPIDLY-GLYCOLYSING SKELETAL MUSCLE

by

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ABBREVIATIONS

C16:0, C18:0, etc.	- fatty acids palmitate, stearate, etc.
CPK	- creatinephosphokinase
DEGA	- diethylene glycol adipate
FFA	- free fatty acids
FSR	- fragmented sarcoplasmic reticulum
GOT	- glutamate-oxaloacetate transaminase
LDH	- lactate dehydrogenase
LPCh	- lysophosphatidyl choline
MH	- malignant hyperthermia
N_1, N_2 and N_3	- fractions of fragmented sarcoplasmic reticulum isolated from slowly-glycolysing muscle and harvested from a discontinuous sucrose gradient, corresponding to bouyant densities of $1{,}184 < \rho < 1{,}210$ g/ml, $1{,}158 < \rho < 1{,}210$ g/ml and $\rho < 1{,}154$ g/ml respectively
P_1, P_2 and P_3	- fractions of fragmented sarcoplasmic reticulum isolated from PSE muscle and harvested from a discontinuous sucrose gradient, corresponding to bouyant densities of $1{,}184 < \rho < 1{,}210$ g/ml, $1{,}158 < \rho < 1{,}184$ g/ml and $\rho < 1{,}154$ g/ml respectively
PCh	- phosphatidyl choline
PE	- phosphatidyl ethanolamine
PSE	- pale, soft, exudative change of post-mortem skeletal muscle of susceptible swine and loosely used to describe both the macroscopc alterations and the biochemical features of accelerated skeletal muscle glycogenolysis
SDS	- sodium dodecyl sulphate
Sph	- sphingomyelin

SUMMARY

1. Fragmented sarcoplasmic reticulum (FSR) was prepared from slowly- and from rapidly-glycolysing porcine skeletal muscle, the latter known to be associated with pale, soft, exudative change (PSE). FSR was fractionated by centrifugation and certain physicochemical functions and characteristics of the various fractions - bouyant density, rate and capacity to accumulate calcium, ATPase activity and its dependence on temperature - were compared. The basis of the differences was sought in terms of lipid components of the FSR membrane.

2. Samples of longissimus dorsi were removed from carcasses of swine within 10-15 min of slaughter by electrical stunning and exsanguination. These were selected as slowly-glycolysing (normal) or rapidly-glycolysing (abnormal) on the basis of colour, consistency, exudation, pH value and lactate and glycogen contents. Rapid glycogenolysis (pH 5,8 at 10-15 min post-mortem) resulted in pale, soft, exudative (PSE) change of the muscle and was correlated with decreased glycogen and increased lactate contents.

3. Crude preparations of FSR from slowly-glycolysing muscle separated into three fractions during centrifugation on a discontinuous gradient of 35, 40 and 45% (^{w}/v) sucrose. The three fractions sedimented at the two interfaces and on the surface of the gradient. The fraction which sedimented at the 40-45% sucrose interface was absent from crude preparations of FSR isolated from PSE muscle. Preparations of crude FSR were also examined by centrifugation in continuous sucrose gradients (15-45% ^{w}/v). From a comparison of the sedimentation behavior it was concluded that FSR from PSE muscle had in general lower bouyant density than normal. The

fractions with lower bouyant density had significantly higher ratios of cholesterol to protein and of phospholipid to protein.

4. The material in each fraction from discontinuous gradients was examined by electron microscopy. All fractions from both normal and from PSE muscle comprised predominantly vesicles consistent in appearance, size and shape with previously described FSR from skeletal muscle of rabbits.

5. FSR which sedimented at the 35/40% sucrose interface ($1,158 < \rho < 1,184$ g/ml) exhibited highest ATPase activity and accumulated calcium at the highest rate and amount. The calcium-accumulating ability of FSR from PSE muscle was approximately 20% of that of normal FSR. In contrast, Mg^{2+}, Ca^{2+}-dependent ATPase activity and the ability of the ATPase to form a phosphorylated intermediate was only slightly impaired.

6. The rate of passive efflux of calcium was measured after the vesicles had been loaded with calcium oxalate in the presence of ATP. The rate constants were similar for normal and for PSE FSR with $t\frac{1}{2}$ values of approximately 30 min. Passive efflux was also measured from vesicles which had been loaded by equilibration with high concentrations of calcium (20 mM) in the absence of ATP. The rate constants for passive efflux after dilution and complexing of calcium with EGTA were similar in both FSR types. Efflux from vesicles isolated from normal muscle was accelerated in the presence of ADP plus Pi. Under similar conditions the rate of efflux from PSE FSR was only slightly enhanced.

7. Passive binding of calcium to FSR membranes was measured by atomic absorption spectrophotometry and the data analysed by Scatchard analysis. Normal membranes possess at least two binding sites with medium affinity

(K_D= 7,6 μM) and low affinity (K_D= 120 μM). The data for the binding of calcium to PSE membranes permitted recognition of only one binding site of intermediate affinity (K_D= 31 μM). Passive calcium binding was also measured by equilibration with ^{45}Ca in the presence of high KCl concentrations (0,1M) which effectively eliminates the non-specific, low-affinity binding site. Under these conditions the calcium-specific sites in normal membranes bind 70-75 nmoles calcium/mg protein and this value corresponds to that of 70 nmoles calcium/mg protein obtained from the Scatchard analysis of the medium-affinity site in the absence of KCl. In the presence of KCl, PSE membranes bind 30-35 nmoles calcium/mg protein as compared to the value of 70 nmoles calcium/mg protein obtained by Scatchard analysis for the single binding site of intermediate affinity. These data suggest that the intermediate site identified in PSE membranes represents a mixture of both calcium-specific, medium-affinity sites and nonspecific, low-affinity sites, but each with diminished capacity.

8. The lipid composition of both whole muscle and FSR membranes was examined in detail. Analysis of lipid extracts of whole muscle revealed that PSE muscle contained less neutral lipid than did normal muscle. Fatty acid analysis of total phospholipid fractions showed no difference in the degree of unsaturation of fatty acyl side chains. The phospholipid fraction was separated into phospholipid classes by means of thin-layer chromatography. The phosphatidyl choline fraction from PSE muscle contained a significantly greater amount of linoleate (25,0-31,9%) and smaller amount of stearate (17,3-9,7%).

9. Purified' FSR membranes contained phosphatidyl choline (60%), phosphatidyl ethanolamine (19%), phosphatidyl inositol plus phosphatidyl serine (11%) and sphingomyelin (5%). A slight but significant decrease from 57 to 46 % in phosphatidyl choline content of the phospholipid fraction from vesicles of lowest bouyant density was the only difference found between normal and PSE FSR.
10. The fatty acid composition of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol plus phosphatidyl serine and sphingomyelin corresponded to that described for FSR from other sources (eg. rabbit). The methylated phosphatidyl ethanolamine fraction contained a large quantity of dimethyl acetals of palmitaldehyde and stearaldehyde and oleoylaldehyde indicating the presence of significant amounts of plasmalogens. The only significant change in fatty acid composition was present in the phosphatidyl ethanolamine fraction in which PSE membranes contained slightly less arachidonate (C20:4ω6) (15,4 versus 12,5%).
11. The protein composition of porcine FSR is qualitatively similar to that of rabbit FSR. The ATPase enzyme is the most abundant protein in the tissue membranes with a mol. wt. of approximately 105 000. Several smaller molecular weight species are present which correspond to similar proteins in rabbit FSR. No consistent differences were noted between the proteins present in the FSR prepared from the two types of muscle, normal and PSE.
12. The temperature-dependence of ATP-dependent Ca-Binding and Ca-Uptake (in the presence of oxalate) was determined and the activation energies of the processes in normal FSR were 10,5 kcal/mole and 22,0 kcal/mole respectively. The activation energy of Ca-Uptake by PSE vesicles was 19,6 kcal/mole.

The temperature-dependence of Mg^{2+}, Ca^{2+}-dependent ATPase activity was determined in the calcium steady-state. An Arrhenius plot of the data from normal membranes had discontinuities at 15°C and 31°C, with relatively high activation energy (29,9 kcal/mole) above 31°C and below 15°C and a low activation energy between these temperatures (12,3 kcal/mole). A similar analysis of ATPase activity of PSE membranes revealed that the process of high-activation energy evident in normal membranes in the temperature range 31-50°C was absent in the abnormal muscle. Temperature-dependence of PSE ATPase in the lower temperature range was similar to that of normal FSR.

Mg^{2+}, Ca^{2+}-dependent ATPase of PSE membranes displayed an increased resistance to thermal inactivation. At 51,5°C, the ATPase activities of both normal and PSE membranes were inactivated in a manner first order with respect to ATPase activity and with rate constants for the inactivation process of 3,5 min^{-1} and 10,0 min^{-1} respectively.

13. Treatment of homogenates of slowly-glycolysing muscle with acid (pH 5,5 for 10 min at 37°C) resulted in decreased calcium accumulation by FSR subsequently isolated from the acid treated homogenate. There was little effect on Mg^{2+}, Ca^{2+}-dependent ATPase activity. Thus the functional changes observed in PSE can be mimicked and most simply explained by the rapid fall in pH in the immediate <u>post-mortem</u> period. The changes in bouyant density noted in FSR of PSE muscle could not be induced in slowly-glycolysing muscle by acid-treatment of the homogenate.

14. The mechanism by which accelerated glycogenolysis is initiated in PSE is still unclear. This study does, however, suggest a mechanism whereby glycogenolysis once initiated could be self-perpetuating and irreversible. It is postulated that the low intracellular pH which occurs in PSE muscle

in the first few minutes post-mortem inactivates calcium transport by sarcoplasmic reticulum. This leads to an accumulation of calcium ions in the sarcoplasm which exceeds levels required to activate phosphorylase b kinase and the myofibrillar ATPase complex ($>10^{-6}$M). Both these processes result in the continued acceleration of anaerobic glycogenolysis, lactate production and a further fall in pH.

The minimal changes in lipid composition of whole muscle and of purified FSR membranes are not considered to be of sufficient magnitude to affect the permeability of membranes of skeletal muscle, susceptible to PSE change. Thus it is unlikely that the increased permeability of these membranes, inferred from the diffusion of enzymes such as CPK out of the cell into the blood plasma of PSE-prone animals can be explained on this basis. It is also considered unlikely that the decreased ability of PSE FSR to accumulate calcium is due to increased permeability of the lipid bilayer to calcium.

15. The results of this study on calcium transport by FSR from PSE muscle permit certain postulates to be made regarding the mechanism of active translocation by this membrane. It appears that nett calcium accumulation can be dissociated from ATPase activity in the absence of increased permeability of the membrane to calcium. This suggests that ATPase activity is not coupled to vectorial transfer of calcium through these deranged membranes. The inability to translocate calcium is accompanied by a fall in the capacity of calcium-specific binding sites of medium affinity and a loss of ATPase activity of high-activation energy above 31°C. These are features which are evident in vesicles with a highly active calcium

transport system, and it is assumed that both are properties of calcium translocation and are not essential during ATP hydrolysis. Since the high-activation-energy ATPase has been shown by Inesi *et al*. (1973) to reflect a conformational change in the ATPase protein, it is postulated that the ATPase in PSE membranes is unable to undergo this conformational change nor that which is thought to occur when calcium translocation is coupled to ATP hydrolysis.

Since the above functional changes can be reproduced *in vitro* by treatment of normal porcine muscle homogenates with acid, this system could be employed to scrutinize mechanisms of active cation transport. Such a study is already in progress.

1.0 INTRODUCTION.

1.0 INTRODUCTION

An insight into mechanisms by which cell membranes actively transport solutes might be gained from a study of conditions under which such transport is deranged. The present work is concerned with a genetic abnormality which affects the skeletal muscle of some animals, particularly some strains of pig, and is characterised biochemically by extremely rapid anaerobic metabolism. Myofibrillar ATPase and the rate-controlling enzymes of anaerobic glycogenolysis are activated. This results in rapid accumulation of lactic acid in affected muscles with consequent physical alterations which ultimately lead to pale, soft and exudative change, the characteristic *post-mortem* expression of the disease. The findings of Ozawa *et al.* (1969), that phosphorylase *b* kinase a vital enzyme in the control of glycogenolysis is activated by a concentration of calcium ions similar to that required by myofibrillar ATPase has provided the intriguing possibility that calcium ions could provide the control that simultaneously activates contraction and energy-regenerative processes. The sarcoplasmic reticulum controls calcium levels in the sarcoplasm and various lines of evidence suggest that its malfunction in susceptible pigs may be responsible for at least some of the changes observed.

An investigation into the biochemical properties of the sarcoplasmic reticulum from altered muscle could provide a model system for examining the normal function of individual components of the calcium pump and for analysing the complex and unsolved problems concerning the transduction of chemical energy into solute translocation through a membrane.

The nature of this investigation - There is evidence that sarcoplasmic reticulum isolated from muscle that has undergone a rapid post-mortem decline in pH has impaired calcium-accumulating ability when compared with that from normal muscle which has a slow rate of pH decline (Greaser et al., 1969a). The enzyme responsible for translocating the cations, namely the ATPase protein, was less affected. The implication is that ATP hydrolysis has been uncoupled from vectorial transfer of calcium through the reticular membrane.

This study establishes the quantitative differences in calcium transport and ATPase activity of fragmented sarcoplasmic reticulum from the two muscle sources and has attempted to determine the nature of the diminished efficiency of sarcoplasmic reticular vesicles from muscles of diseased animals. In view of the membrane-bound nature of the pump protein, the influence of the lipid environment on its functional activity was considered and a detailed analysis of the lipids of the sarcoplasmic reticular membrane was carried out. The lipid studies included an analysis of whole muscle since a general derangement of muscle cell membranes is inferred in this disease from the diffusion of proteins and ions out of the muscle cell into the extracellular fluid. In addition, the nature of the lipid-ATPase interaction was examined by observing the effect of temperature on the functional activities of the sarcoplasmic reticulum.

Limitations of this study - The choice of the model does not allow any conclusions to be drawn concerning the functional and compositional status of the sarcoplasmic reticulum *in vivo*. The syndrome is most easily recognised by the physical and biochemical changes in *post-mortem* skeletal muscle and hence it cannot be excluded that alterations subsequently found in affected muscle were due to *post-mortem* effects. Since genetically predisposed pigs normally show no outward manifestations of the disease it can be presumed that the calcium-accumulating ability of the sarcoplasmic reticulum functions normally during life. What has been clearly shown, however, is that within a short period of death the severity of the defect arising from autolytic changes in the tissue, is such that it could explain how glycogenolysis, once activated in susceptible skeletal muscle, rapidly becomes irreversible and self-perpetuating.

1.1 THE SYNDROME OF PALE, SOFT, EXUDATIVE MUSCULATURE.

With the selection of a meatier and more muscular type of domestic pig for breeding purposes, certain breeds such as the Landrace of South Africa, Australia, Denmark and England, the Pietrain of Holland and the Poland China of America have developed an intolerance to stress which very often proves fatal (Briskey 1964; Briskey and Lister, 1968; Bickhardt *et al.*, 1972; Patterson and Allen, 1972). There are thus an increasing number of deaths during transportation of these animals and just prior to slaughter, circumstances which promote excitement and struggling. On the other hand, breeds such as the Chester White and Large White which have less muscle bulk, are stress-resistant.

If stress-susceptible pigs die as a result of stress or are stressed during slaughter, then certain muscles, *post-mortem*, have a pale appearance, soft texture and watery consistency (Wismer-Pederson and Briskey, 1961; Bendall and Lawrie, 1964; McLoughlin and Goldspink, 1964). In addition *rigor-mortis* develops exceptionally rapidly (Bendall, 1966). Accelerated glycogenolysis, glycolysis and phosphate ester hydrolysis in the skeletal muscle are associated with these altered physical characteristics.

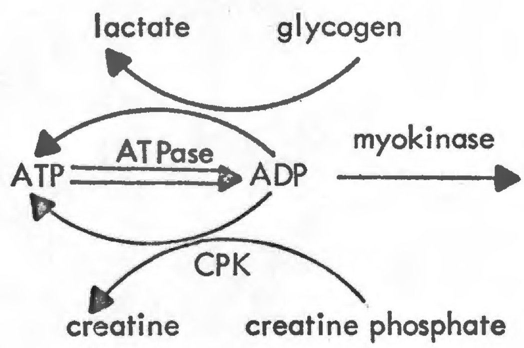
The muscles which are most affected are the so-called white muscles, such as *longissimus dorsi*, *semimembranosus* and *biceps femoris*. These have been especially adapted for rapid muscular activity over a short period and have a high capacity for anaerobic glycogen degradation via glycolysis, both with respect to the large glycogen stores present in the muscle cell and in the high phosphorylase activity, which is about five times that of red muscle.

Characteristically, glycogen is broken down and lactic acid rapidly accumulates in the post-mortem muscle - which ultimately becomes pale, soft and exudative, often to the extent of 100 µmoles per gram tissue within a few minutes after death and within half an hour the pH can be as low as 5,1, whereas muscle from stress-resistant animals only reaches pH 5,5 12-24 hours post-mortem (Briskey et al., 1959; Briskey, 1964; Wismer-Pedersen, 1959). The rapid accumulation of lactic acid lowers the pH to below 6,0 while the carcass temperature is still above 35°C. The combination of low pH and high temperature is thought to adversely affect meat quality and cause it to become PSE (the term will be loosely used in the rest of this dissertation to indicate both macroscopic changes and accelerated glycogenolysis). Briskey and Wismer-Pedersen (1961), correlated the rate of post-mortem decline in pH with the ultimate condition of the muscle. The most marked changes observed to accompany the rapid pH decline are denaturation of sarcoplasmic proteins and their resultant insolubility (Sayre and Briskey, 1963). It appears that these proteins precipitate in the Z line region of the muscle cells and possibly on the myofibrils as well (Greaser et al., 1969 b). The loss of water-binding capacity is probably related to this change (Bendall and Wismer-Pedersen, 1962). The pale colour is not the result of leakage of myoglobin into the extracellular fluid (Briskey and Kauffman, 1971), as perhaps would be expected, but appears to arise from an alteration of physical properties of the surface layers of tissue with the result that light transmission is decreased whilst reflectance of surface layers of the muscle tissue is enhanced and the muscle appears paler than usual.

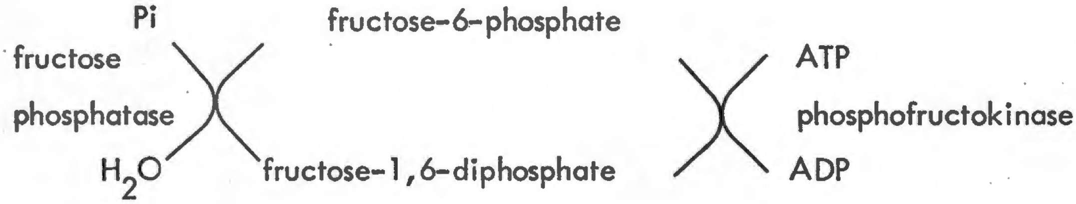
The rate of post-mortem hydrolysis of ATP in these muscles is greatly increased over that of normal white muscles. Heffron and MacLoughin (1971), obtained a rate of 16,3 μmoles ATP hydrolysed/g of tissue. $\min^{-1}$ during the first eight minutes post-mortem as opposed to a control value of 0,7 μmoles ATP hydrolysed/g tissue. $\min^{-1}$. They calculated that, of the three common ATPases known to be functional in muscle, namely the mitochondrial, sarcoplasmic reticular and myofibrillar ATPases, the high initial activity could only be accounted for by the Mg^{2+}-stimulated myofibrillar ATPase, the enzyme involved in muscular contraction. Under normal physiological conditions the ATP level in muscle does not fluctuate to any measurable extent as the rates of ATP synthesis by anaerobic glycolysis and oxidative phosphorylation are closely controlled and regulated according to energy utilisation, eg muscular contraction. In the condition of PSE, normal control mechanisms appear to be operative. Although glycogenolysis and glycolysis appear to be accelerated to near maximal levels achieved during physiological muscle contraction, creatine phosphate concentrations decline. AMP accumulates to maximal levels at 30 min after slaughter after which the level declines (Kastenschmidt, 1970). This later catabolism of AMP is most likely due to the high activity of adenylic deaminase in muscle tissue. This enzyme deaminates AMP to IMP. The accumulation of AMP and Pi as a result of hydrolysis of ATP and myokinase activity has significance in the stimulation of those enzymes in the glycolytic pathway which catalyse non-equilibrium reactions (see below).

Biochemical changes similar to those provoked by stress in the white muscles of stress-susceptible pigs can be induced in the same animals and in certain humans by exposure to anaesthetic agents such as halothane ($CF_3CHBrCl$) and succinyl choline (Sybesma and Eikelenboom, 1969; Berman et al., 1970; Britt and Kalow, 1970). The syndrome, triggered by these agents is characterised by a rapid rise in body temperature, severe lactocidosis and rigor of skeletal muscle and is known as malignant hyperthermia (MH) or malignant hyperpyrexia. The former and its abbreviation, MH, is preferred and will be used in the ensuing text. It is thought to be an expression of the same fundamental abnormality as the PSE syndrome although muscle from animals that have died as a result of a malignant hyperthermic episode exhibits less obvious pale, soft and exudative change (Berman and Kench, 1971). These authors suggest that the reason may be because animals that have died from exposure to halothane are not usually exsanguinated immediately post-mortem as are pigs slaughtered at abattoirs. The presence of blood in the circulatory system may allow sufficient lactate to diffuse out of the muscle and slow the rate of pH decline in the sarcoplasm during the crucial minutes at death when the body temperature is still high. Berman and Kench (1971) investigated the changes in adenine nucleotides and creatine phosphate during the induction of MH by halothane in MH-susceptible pigs. Within 2 min of introduction of halothane into the anaesthetic circuit after a control period of pentothal anaesthesia creatine phosphate level declined significantly. During the 10 min that halothane was administered when glycogenolysis and glycolysis were maximally accelerated, creatine phosphate virtually disappeared whilst ATP level showed an initial increase but after 10 min also declined.

During this period ADP and AMP concentrations were unaltered but after 15 min IMP accumulated. It was suggested that these findings were due to acceleration of glycogenolysis and glycolysis and of myofibrillar ATPase and that the transient initial rise in ATP concentration was due to an imbalance between glycolytic generation of ATP and its hydrolysis by myofibrillar ATPase. The accumulation of IMP was thought to result from adenylate deaminase activity according to the following reaction scheme.



Although Heffron and MacLoughlin calculated that only the myofibrillar ATPase could account for the high rate of hydrolysis of ATP, Lardy's group (Clark _et al._, 1974) have demonstrated the existence of an accelerated substrate-cycling process in halothane-susceptible pigs which in effect acts as an ATPase. Fructose-6-phosphate is phosphorylated to fructose-1,6-diphosphate by phosphofructokinase and subsequently dephosphorylated to fructose-6-phosphate by fructose diphosphatase.



The nett result of such cycling is the hydrolysis of ATP to ADP and Pi. They showed that a 30-80 fold difference in the rates of substrate cycling was evident between sensitive and insensitive animals when exposed to halothane. Whether this cycle also operates in the PSE syndrome has not yet been established.

Stress-susceptibility and malignant hyperthermia are associated with a diffusion of enzymes and cations from the muscle cell into the extracellular fluid. Bickardt (1971) found that stress-susceptible animals have raised plasma CPK, GOT and ALD activities at all stages of growth and the release of these enzymes from the myoplasm was increased following exertion. CPK has unusually high activity in the plasma and has been used to predict stress-susceptibility in pigs (Allen and Patterson, 1971; Berman, et al., 1971) malignant hyperthermia in pigs (Woolf et al., 1970) and in humans (Isaacs and Barlow, 1970). LDH also leaks out of the muscle cells following a hyperthermic episode (Sybesma and Eikelenboom, 1969). Considerable amounts of K^+, Mg^{2+}, Na^+ and Pi accompany the extrusion of these enzymes during hyperthermia (Berman et al., 1970). Schmidt et al. (1970), found significant increases in serum Ca^{2+}, Na^+ and Pi in stress-susceptible (Poland China) pigs when compared to the levels found in stress-resistant (Chester White) pigs following death by captive bolt pistol.

1.2 THE AETIOLOGY OF PALE, SOFT, EXUDATIVE CHANGE.

During the catabolism of glycogen to lactic acid three enzymes catalyse non-equilibrium reactions, namely phosphorylase, phosphofructokinase and pyruvate kinase and of these it is likely that the major control point in the pathway exists at the level of the enzyme phosphorylase with its complex regulation by metabolites, ions and enzymes (Newsholme and Start, 1973).

Phosphorylase has two forms, namely phosphorylase a and phosphorylase b, which are enzymatically interconvertable. (Fischer and Krebs, 1955).

$$\text{phosphorylase } \underline{a} + 4\ H_2O \xrightarrow{\text{phosphorylase phosphatase}} 2 \text{ phosphorylase } \underline{b} + 4\ Pi$$

$$2 \text{ phosphorylase } \underline{b} + 4\ ATP \xrightarrow{\text{phosphorylase } \underline{b} \text{ kinase}} \text{phosphorylase } \underline{a} + 4\ ADP$$

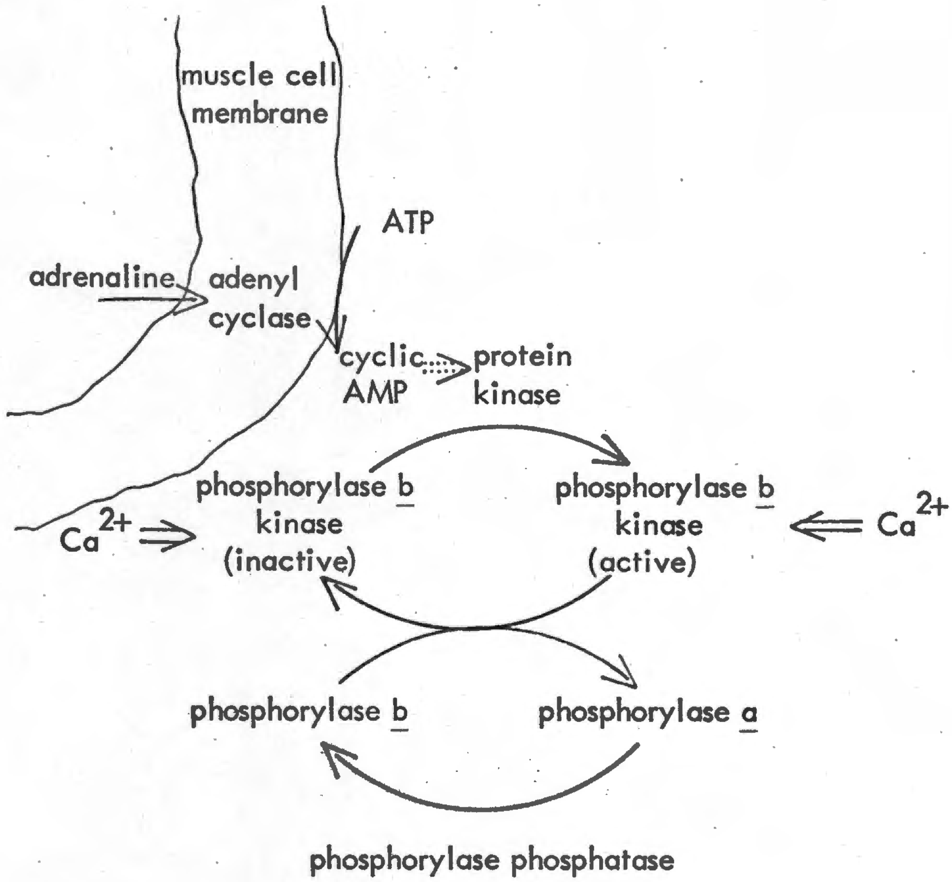
Phosphorylase a is always active whereas phosphorylase b becomes active if the ratio (AMP + Pi)/(ATP + G-6-P) is relatively high. In resting muscle the enzyme is in the b form and, as the above ratio is low, glycogen is only slowly converted to G-1-P. Activation of phosphorylase can occur either by increasing the ratio or by converting phosphorylase b to a. The latter method thus allows glycogenolysis to be accelerated despite low levels of AMP and Pi.

The first mechanism is the more primitive; high AMP and Pi levels also stimulate phosphofructokinase. As has been mentioned, there is an accumulation of AMP and Pi in the fast-glycolysing muscles that become PSE and would serve to activate phosphorylase b. However, whether high levels of AMP and Pi provide the initial stimulus cannot as yet be ascertained. Conditions that could result in a high ratio of (AMP + Pi)/(ATP + G-6-P) are those in which either the supply of ATP is not sufficiently fast, the production of ATP by the mitochondria is abnormally slow, or the ATPase systems abnormally active.

Uncoupling of oxidative phosphorylation, as a cause of the accelerated glycogenolysis, was proposed by Sybesma and Eikelenboom, (1969). Wilson et al. (1966) and Gatz, (1973) have proposed that the action of halothane in triggering a malignant hyperthermic episode was through a similar mechanism. However both Wang et al., (1969), on the basis of thermodynamic data and Berman et al., (1970), on calculations of O_2 consumption during a hyperpyrexic crisis, have concluded that the heat produced cannot be accounted for by uncoupling of oxidative phosphorylation but rather arises from anaerobic metabolism. Work by Harris et al. (1971), and Berman and Colleagues (Berman et al. 1974; Kewley et al. 1972), showed that the effect of halothane on mitochondrial function was rather to inhibit NADH dehydrogenase, resulting in reduced O_2 consumption and ultimately lessened ATP synthesis. Eikelenboom and coworkers have now abandoned the uncoupling hypothesis and have demonstrated a lower O_2 consumption in isolated mitochondria of stress-susceptible pigs compared to those from normal animals.

Besides aberrant mitochondrial function, a decreased ATP production could be the result of diminished oxygen supply to the PSE-prone muscles. The adrenergic response during stress, representing a release of noradrenalin and adrenalin, could cause a constriction of the smooth muscle in the micro-vascular system. However, blocking the response of α receptors in the vascular smooth muscle and stimulating the β receptors to active dilation has minimal effect (Lister _et al._, 1970). Stress-resistant pigs forced to breathe 100% O_2 or N_2 accumulated no lactate in their muscles whereas stress-susceptible animals did on breathing either gas although nitrogen had a more marked effect. Lister and coworkers advanced the suggestion that such pigs may be particularly susceptible to anoxia.

The more complex mechanism of stimulating glycogenolysis is to convert phosphorylase _b_ to _a_. The reaction is performed by the enzyme phosphorylase _b_ kinase which itself can exist in an active and inactive form. Phosphorylation of the inactive form by protein ;kinase converts it into the active form. In converting phosphorylase _b_ to _a_ the active kinase enzyme reverts back to the inactive species.



The protein kinase is activated by cyclic AMP which arises from the action of adrenaline on adenyl cyclase in the membrane of the muscle cell. Thus, adrenaline circulating in the bloodstream is a potent stimulator of glycogenolysis and causes a build up of hexose phosphates and triose phosphates, which are the immediate precursors for ADP phosphorylation, in preparation for muscular activity (Newsholme and Start, 1973). However, without an activation of hydrolysis of ATP no lactate is formed. Judge et al. (1968), who measured the amount of catecholamines in the urine of stress-susceptible and stress-resistent animals found excretion levels were unrelated to the post-mortem properties observed.

Aberle and Merkel (1968) found that injecting adrenaline in pigs 10 min prior to slaughter did not significantly alter the quality of the pork.

Both the active and the inactive forms of phosphorylase b kinase are activated by Ca^{2+} at the same concentration that provokes muscular contraction (Ozawa et al., 1967). Nervous excitation promotes the release of Ca^{2+} from the sarcoplasmic reticulum (SR) ~~and the concentration~~ of Ca^{2+} in the sarcoplasm increases over the approximate range 10^{-8} - 10^{-6}M. This is sufficient to activate the myofibrillar ATPase and initiate contraction. Relaxation is brought about by the accumulation of Ca^{2+} by the reticulum so that the concentration in the sarcoplasm returns to 10^{-8}M. Ebashi and his coworkers found that phosphorylase b kinase is activated by Ca^{2+} in the concentration range 10^{-7} - 10^{-6}M, and further work by Krebs and colleagues (Brostrom et al., 1971) established that both active and inactive forms are stimulated in this concentration range. The cyclic AMP-protein kinase system is by-passed, phosphorylase b is converted to phosphorylase a and glycogenolysis is stimulated at the same time as contraction is initiated.

If the concentration of Ca^{2+} in the sarcoplasm is somehow maintained at about 10^{-6}M as a result of stress in stress-susceptible animals, then both accelerated glycolysis and rigor observed in these animals could have a common explanation. The presence of Ca^{2+} in such a concentration could accrue through an inability of the SR to lower the sarcoplasmic Ca^{2+} concentration, either as a result of defective SR function or of abnormal nervous stimulation.

The latter possibility was followed up by Schmidt et al. (1972), and they found that the resting membrane potential of muscle from stress-susceptible Poland China pigs was 20–25mV lower at 45 min post-mortem than that of stress-resistent Chester White pigs. This suggests that electrolyte readjustment was faulty, reflecting either augmented permeability or defective K^+, Na^+-ATPase pump. The hyperirritability of these animals during life may be a manifestation of such a defect. Swatland and Cassens (1972) noted excessive ramification and growth of the myoneural and plate junction. However, these differences have not been correlated with elevated Ca^{2+} levels and their significance is difficult to establish.

A malfunctioning SR has been increasingly cited as the cause of both stress-susceptibility and malignant hyperthermia. Although Greaser et al. (1967), have shown that isolated SR loses its Ca^{2+}-accumulating ability relatively rapidly after death in porcine muscle during a normal pH decline, Greaser et al. (1969a) demonstrated that fragmented sarcoplasmic reticulum (FSR) isolated from fast-glycolysing muscle loses it even quicker, the difference at 5 min post-mortem, approaching significance, at half an hour being well established. In the first hour post-mortem, Ca^{2+}-stimulated ATPase activity, responsible for the active transport of Ca^{2+} into FSR, did not change from that of controls. The effect of the combination of low pH and high temperature, is difficult to assess but must be taken into consideration since Greaser et al. (1969c), have shown that incubation of FSR at pH 5.5 and 37°C for an hour completely destroys the Ca^{2+}-accumulating ability. In order to eliminate post-mortem effects, Greaser et al. (1969e), measured Ca^{2+} transport in FSR isolated from biopsied muscle and from muscle taken at the time of death and found both to have significantly lower activity as compared to normal controls.

Kalow et al. (1970), reported that muscle preparations from human survivors of anaesthetic-induced malignant hyperthermia were more sensitive to caffeine with onset of rigor, than that of normal controls and this effect was enhanced by exposure to halothane. Halothane depressed the accumulation of Ca^{2+} by the SR from these patients whereas it had no effect on that from normal people (confirmed by Brucker, 1971, and Denborough et al., 1971). Caffeine acts by interfering with the normal function of the SR membrane, causing a release of Ca^{2+} from the SR (Weber,1968) and it has been suggested that caffeine-induced contracture might be used as a model for studying malignant hyperthermia (Strobel and Bianchi, 1971).

Procaine added either before or after exposure of susceptible muscle to halothane consistently inhibited contracture and blocked the initiation of the syndrome in live animals (Moulds and Denborough, 1974; Harrison, 1973). This agent acts on the SR membrane by preventing Ca^{2+} efflux from the SR.

On the basis of these experiments many researchers believe the primary defect to reside within the SR.

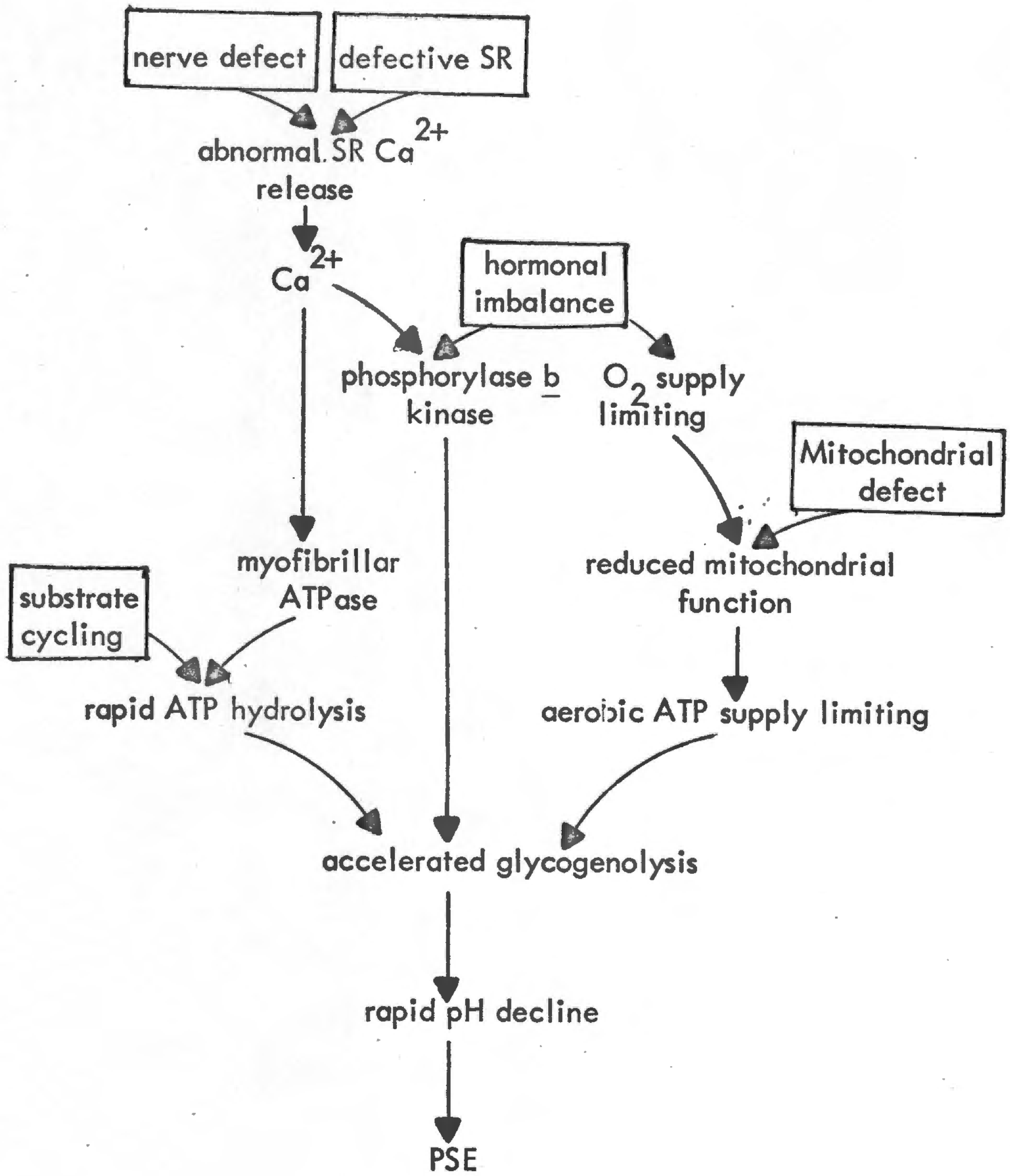


fig 1 The possible origin of PSE

1.3 THE PHYSIOLOGICAL ROLE OF THE SARCOPLASMIC RETICULUM.

The SR consists of a flattened double-membrane vesicle with many perforations, surrounding each set of adjacent sarcomeres in a myofibril. In the muscles of the frog and mammal each extends from the A-I junction of one sarcomere to that of the next. The internal compartment or cisterna of each vesicle is connected to all others in the same cross striation by transversely-orientated connecting channels called terminal cisternae. Pairs of parallel terminal cisternae run across the myofibrils in close contact with the transverse tubules of the T-system (Franzini-Armstrong, 1964; Constantin et al., 1965; Porter and Palade, 1957). (See fig 2).

The SR controls contraction and relaxation in skeletal muscle by alternatively raising and lowering the Ca^{2+} content of the sarcoplasm.

Contraction is initiated when the accumulation of Ca^{2+} in the sarcoplasm reaches a threshold value of $0,7 - 1,5 \times 10^{-6}$M (Portzehl et al., 1964). The increase of Ca^{2+} in the sarcoplasm is the end result of depolarisation of the surface membrane and the conduction of the action potential into the interior of the muscle along the T-system whose interior is continuous with the extra-cellular space (Franzini-Armstrong and Porter, 1964; Huxley, 1964). The closely apposed membranes of the terminal cisternae are altered in some way and a trigger release of Ca^{2+} into the sarcoplasm results. The nature of the link between the T-system and the terminal cisternae is not clear, but is thought to be electrical (Constantin and Podolsky, 1966). A relatively small voltage (a few mV) across the membrane of the SR causes release of Ca^{2+}.

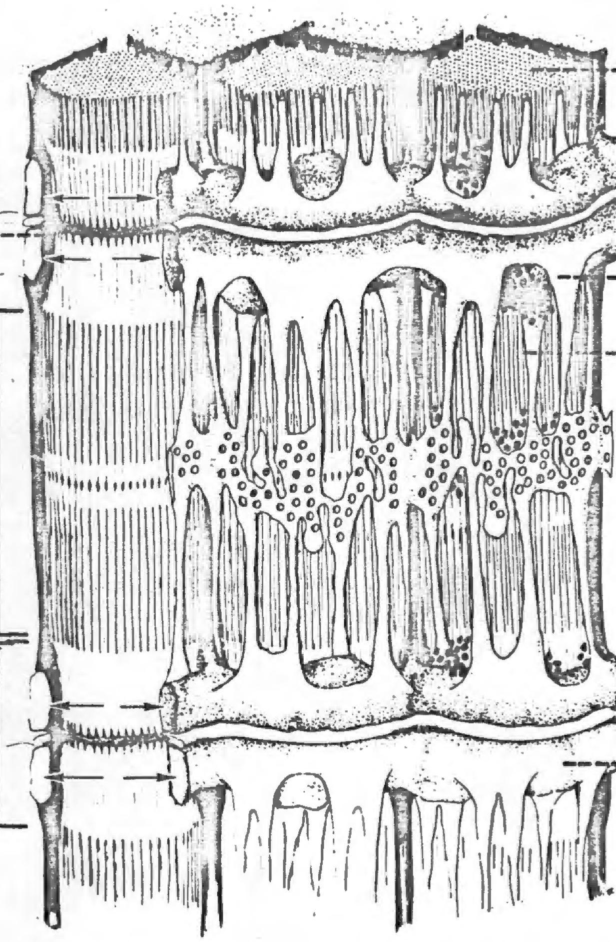


Fig. 2 <u>Location of the sarcoplasmic reticulum within the muscle cell.</u>
(Peachey, 1965, <u>25</u>, 209-231)

Since depolarization of about 50 mV at the sarcolemmal membrane is necessary to initiate contraction, only about one tenth of the T-system potential needs to be transfered across to the terminal cisternae for release of Ca^{2+} (Constantin and Podolsky, 1966, 1967). The possibilities of a chemical transmitter, although less likely, have not been excluded (Huxley, 1971).

Autoradiographic studies by Winegrad (1965) showed that in resting muscle Ca^{2+} is localised primarily within the terminal cisternae and it is thought that Ca^{2+} is released from there into the sarcoplasm. The specific mechanism of mediation and actual release of Ca^{2+} from the store remains still unknown.

The regulatory nature of the action of Ca^{2+} on the contractile system is being intensively studied (Cold Spring Harbour Symposium, 1973). It has been established that the sensitivity of the contractile system to Ca^{2+} resides neither with myosin nor actin but with a complex of proteins called troponin located along the actin polymer. This complex consists of three proteins, an inhibitory protein which can inhibit the Mg^{2+}-stimulated ATPase of actomyosin and essentially prevents actin and myosin interaction, a Ca^{2+}-binding protein which has a high affinity for Ca^{2+}, and a third which has an unknown function (Perry et al., 1973). When the Ca^{2+} concentration is raised to about 10^{-5}M in the sarcoplasm, the Ca^{2+}-binding protein binds Ca^{2+} and in so doing neutralises the action of the inhibitory protein, allowing actin and myosin interaction, and hence contraction. The manner in which this is done is unknown but it seems that a change in the conformation of the troponin complex alters the actin monomers through mediation of tropomyosin, another protein which lies in each groove of the double strand of actin and which is in contact with both (Bremel and Weber, 1972).

Relaxation is achieved by decreasing the sarcoplasmic Ca^{2+} concentration to a resting level of about 10^{-7} M and this is done by the active accumulation of Ca^{2+} into the SR. From autoradiographic results and less direct evidence such as the heterogeneity of the SR and the delay between accumulation and release of Ca^{2+} Winegrad (1965), proposed that Ca^{2+} is accumulated at a place different from where it is released. He suggested that a cycle exists in which the longitudinal cisternae sequester Ca^{2+} which subsequently diffuses or is transported back to its release site in the terminal cisternae. However both fragments of the reticulum are able to accumulate Ca^{2+} (Hasselbach, 1964).

The data obtained from isolated fragmented SR (FSR) can explain the resting state of muscle, ie, the Ca^{2+}-binding capacity of FSR is capable of lowering the Ca^{2+} concentration of the sarcoplasm below the threshold level necessary for contraction (Ebashi and Endo, 1968). It has also been determined, using a calcium-sensitive dye, murexide, and Chance's stopped-flow technique, that the speed of Ca^{2+} binding to the FSR, sufficient to cause relaxation, is of the same order as the speed of relaxation. They have concluded that the results obtained with isolated FSR could account for the events in living muscle.

SR is poorly represented in heart muscle (Fawett and McNutt, 1969) and shows diminished ability to accumulate Ca^{2+} in vitro (Harigays and Schwartz1969). This has led to an investigation of the contribution by other organelles or membranes to the control of Ca^{2+} levels in the myoplasm. Both the mitochondria (Lehninger, 1970) and the sarcolemma (Yamamoto, 1967) have been implicated with the latter probably having the more important role.

1.4 THE STRUCTURE AND COMPOSITION OF THE SR MEMBRANE.

During isolation of SR membranes they become fragmented and resealed to form vesicles between 600 to 3000 Å in diameter and enclosed in membranes 60-70 Å thick (Nagai et al., 1960; Ebashi and Lipman, 1962). The interior of a vesicle is either empty or contains a poorly-defined filamentous network (Baskin and Deamer, 1969).

SR membranes are highly specialised structures with one clearly identified function, the regulation of sarcoplasmic Ca^{2+} concentration and this is reflected in the relative simplicity of the membraneus composition. Transport ATPase constitutes 60-80% of the protein content of the membrane. The enzyme has an absolute dependence on phospholipids for activity. This points to it being lodged within the hydrophobic interior of the membrane and, in fact, freeze-fracture electromicrographs reveal globules about 75-100 Å in diameter in this region (Deamer and Baskin, 1969). These globules have a similar structure to isolated ATPase protein (MacLennan et al., 1971, 1972) and are present in membranes prepared from the ATPase protein and phospholipids, although absent in membranes composed of phospholipids alone. Further evidence that the globules are composed of ATPase proteins comes from the study by Tillack et al., (1974) of developing chick embryos. A sharp increase in Ca^{2+} transport and Ca^{2+}-sensitive ATPase activity is accompanied by an increase in the number of these globules.

Negatively-stained (electron micrographical) preparations show vesicles surrounded by a granular layer, made up of projections 30-40 Å in diameter and 60 Å apart.

Very similar projections are observed in vesicles formed artificially from purified ATPase by removal of detergent from solubilised preparations of the enzyme (Stewart and MacLennan, 1974). The projections are destroyed by high concentrations of trypsin alongside loss of ATPase activity. Thorley-Lawson and Green (1973) found that, with lower levels of trypsin, ATPase is split into two fragments of very similar size (60 000 and 55 000 mol wt.) which remain attached to the membrane. The 60 000 entity is slowly split into fragments of 33 000 and 24 000. The 33 000 component was shown to contain the phosphorylated active site. Both fragments were extensively iodinated by lactoperoxidase, indicating that they were exposed on the outer surface and responsible for the observed electron micrographical projections. The 55 000 component could not be iodinated and must be the hydrophobic part of the ATPase molecule which is buried in the membrane and appears as the globules seen in freeze-fracture micrographs. Essentially similar results were obtained by Stewart and MacLennan, 1974. Thorley-Lawson and Green found that the 55 000 component, which was not broken down by trypsin, could not be iodinated even when both faces of the membrane were accessible to labelling reagents. If the ATPase does span the width of the membrane, it does not expose any significant amounts of iodinable tyrosine nor trypsin-sensitive bonds in the region.

Several other acidic proteins can be detached more easily from the membrane than can ATPase, e.g. by chelation with EDTA. Calsequestrin (44 000) has the ability to bind large quantities of Ca^{2+} (30-40 ions/molecule) at rather low affinity in the presence of 0,1 M KCl. It is thought by MacLennan (MacLennan and Wong, 1971) to play a role in calcium binding in the reticulum but the finding of Thorley-Lawson and

Green (1973) that it may be located on the exterior of the membrane has made its proposed function as an internal Ca^{2+} store less likely. Another acidic protein (55 000) binds Ca^{2+} with a high affinity but with low capacity (1 ion/molecule) (Ikemoto et al., 1974; Ostwald and MacLennan, 1974). Three other acidic protein (24 000 - 38 000) bind Ca^{2+} but with a low affinity. (MacLennan and Wong, 1971).

Approximately 40% of the dry weight of the membrane is accounted for by lipid and of this about 90% consists of phospholipid (Marai and Kuksis, 1973; Meissner and Fleischer, 1971). The major phospholipid is phosphatidyl choline followed by phosphatidyl ethanolamine. Phosphatidyl inositol and phosphatidyl serine, sphingomyelin and cardiolipin are also present but in smaller quantities. Significant amounts of alkenyl acyl derivitives have been found in the phosphatidyl choline and phosphatidyl ethanolamine fractions. (Waku et al., 1971; Owens et al., 1972). The neutral lipids, comprising only 10% of the total lipids, consist mainly of cholesterol but contain small amounts of triglyceride and free fatty acids (Marai and Kuksis, 1973).

Each phospholipid has a characteristic pattern of fatty acids associated with it, for example there are high ratios of palmitic acid: oleic acid and palmitic acid: linoleic acid in phosphatidyl choline whereas the most common phosphatidyl ethanolamine species has high stearic acid: arachidonic acid (Marai and Kuksis, 1973).

The general structure of the SR membrane conforms to that portrayed in the lipid-globular protein mosaic model originally proposed by Lenard and Singer (1966) and later substantiated by Glaser et al. (1970) and Singer and Nicolson (1972).

In this model, the lipids and globular integral proteins (in this case the ATPase enzyme) were arranged in an alternating mosaic pattern throughout the membrane. The hydrophobic portions of the lipid and most of the nonpolar amino acid residues of the proteins were buried in the hydrophobic interior of the membrane, out of contact with water. The ionic or polar groups of the phospholipids and the charged residues of the proteins aligned themselves on the surface of the membrane in association with water. Thermodynamically this is an extremely stable arrangement, maximising hydrophobic and hydrophilic interactions.

In this mosaic model, the lipids were predicted to be primarily arranged in the form of a bilayer, similar to that proposed by Davson and Danielli in 1952. The theoretical width of a bilayer is about 40-45 Å whereas the SR membrane is 60-70 Å. This difference could be explained either by the presence, between the lipid monolayers, of substantial amounts of neutral lipids which causes the membrane to expand or the width of the SR membrane is an average value and includes the projections of the ATPase molecule and the other extrinsic proteins, into the aqueous phase.

The physical state of a membrane is to a large extent determined by the chain length and degree of unsaturation of the fatty acid side chains of the phospholipids (Rottem _et al._, 1970). The presence of _cis_ double bonds in the fatty acid side chains disrupts the orderly stacking of the phospholipids in a membrane causing it to be more fluid. Short chain fatty acids have the same effect.

The importance of the physical state of the membrane has been established with experiments on Mycoplasma laidlawii and it appears that the fluidity of a membrane must be maintained for normal function (McElhaney, 1974). The permeability of a membrane is directly related to physical state as determined by the geometric configuration (cis and trans) and number of double bonds present in the fatty acyl chains (McElhaney et al, 1970). Spin label and NMR studies have emphasised the fluid nature of the SR membranes. (See later).

1.5 THE MECHANISM OF CALCIUM ACCUMULATION, STORAGE AND RELEASE BY SARCOPLASMIC RETICULUM.

In the presence of Mg^{2+} and ATP, Ca^{2+} rapidly becomes associated with fragmented SR, to the extent of 120–150 nmoles Ca^{2+}/mg protein (rabbit FSR) by a mechanism designated as Ca-binding (Ebashi, 1961; Hasselbach and Makinose, 1961; Weber, et al., 1966). If oxalate is included in the reaction medium, such that the concentrations of Ca^{2+} and oxalate in the medium are kept below the solubility product of calcium oxalate, then at least ten times more calcium is accumulated by the vesicles, although at a slower rate (Martonosi and Ferretos, 1964; Hasselbach and Makinose, 1961). The latter process is termed Ca-Uptake. Martonosi and Ferretos showed that the effect of oxalate is to prevent the levels of Ca^{2+} accumulated within the vesicles becoming rate limiting, through the intravesicular precipitation of calcium oxalate. Electron micrographs clearly reveal the precipitates within the vesicles (Greaser et al., 1969d). In both processes translocation of calcium occurs at the expense of ATP.

The membrane fragments sustain a low rate of hydrolysis of ATP in the absence of Ca^{2+} (termed 'basal' ATPase activity). On addition of Ca^{2+}, additional or 'extra' splitting of ATP takes place, which maintains a fairly constant rate until maximal binding or uptake has been achieved. At this stage the rate drops to a steady state which is thought to represent a balance of Ca^{2+} efflux from and Ca^{2+} influx into the vesicles (Hasselbach and Makinose 1963; Hasselbach, 1964; Weber, 1971).

Under optimal conditions, a ratio of two to one between Ca^{2+} accumulated in the presence of oxalate and 'extra' ATP hydrolysed can be demonstrated (Hasselbach and Makinose, 1963). However, discordent results are often obtained in the absence of oxalate eg. Ebashi and Yamamouchi (1964) and this process might accumulate Ca^{2+} via a different mechanism. The fact that Ca-Uptake and 'extra' splitting of ATP are strictly correlated suggests that the energy of ATP hydrolysis was used for transporting Ca^{2+} through the membranes.

Because Ca-Binding is much more rapid (40 nmoles Ca^{2+}/mg protein can be bound in 30 msec) than Ca-Uptake, Ca-Binding might represent an early step in the process that affects Ca-Uptake. Alternatively, as suggested above, they could represent two independent mechanisms. Katz and Repke (1973) found that Ca-Binding follows saturation kinetics whereas Ca-Uptake does not, provided the measurements are done below inhibitory levels of Ca^{2+}. In the range of Ca^{2+} concentration within which Ca-Binding is saturated, the rate of Ca-Uptake increases linearly with increasing Ca^{2+} concentration. Either the binding sites involved in Ca-Binding do not function during Ca-Uptake under these conditions or another step in the process of Ca -Uptake is rate limiting.

Entman and coworkers (Entman et al., 1973; McCollum et al., 1972) working with cardiac FSR found that the time course of Ca-Uptake could fit a curve computed for a two-component system better than one for a one-component system and designated two sites, which could be involved in Ca -Uptake, A and B. The sites differed in affinity for Ca^{2+}, requirements for Mg^{2+}, pH dependance and the effect of ionophores and arsenate.

The influence of these parameters and compounds on Ca-Uptake exactly paralleled their effect on site B, which suggested that this site is associated with the uptake process and is probably located on the interior of the membrane. Ca-Binding primarily involves site A. During Ca-Uptake, Ca^{2+} is first bound to site A and then spontaneously released and attached to site B. They were able to demonstrate that Ca-Binding is followed by a spontaneous release phase. Such a phase has not been demonstrated in skeletal muscle FSR.

Studies on energised transport of Ca^{2+} into intact SR vesicles by what is probably a more physiological process of Ca-Binding reveals a K_m of 0,2-0,5 μM (Weber et al., 1966; Ebashi and Endo, 1968; Carvalho and Leo, 1967; Katz and Repke, 1973). Passive binding of Ca^{2+} to FSR should enable a distinction to be made between high- and low-affinity binding sites, and Ca^{2+}-specific and Ca^{2+} non-specific sites. The results, however, are confusing. In general, it is agreed that at least one site exists which is Ca^{2+}-specific, with a capacity of 5-20 nmoles/mg protein and a K_D of 1-40 μM (Carvalho, 1966; Cohen and Selinger, 1969; Chevalier and Butow, 1971; Meissner et al., 1973). A second site has been recognised by some workers to be Ca^{2+}-specific (Chevalier and Butow, 1971) and by others (Cohen and Selinger, 1969) Ca^{2+} non-specific, with a capacity of 45-130 nmoles Ca^{2+}/mg protein and a K_D of 0,5-45 μM. A third non-specific site was found by Chevalier and Butow in the absence of KCl, with a capacity of 850 nmoles Ca^{2+}/mg protein and a low affinity.

Study of the individual protein components isolated from the membrane has indicated that the Ca^{2+}-specific, low capacity but high affinity site is located on the ATPase molecule (MacLennan et al., 1971; Meissner, 1973). Ikemoto, 1974, has recently reported another two non-specific sites on the purified ATPase protein but the data indicate that they do not play a role in translocation of Ca^{2+}.

Only one other protein possessing a calcium specific binding site has been isolated and that is a 55 000 dalton component with a K_D of 3 μM and capacity of 18 nmoles Ca^{2+}/mg protein. The other five proteins found by MacLennan to constitute the SR membrane can all bind Ca^{2+} with low, but differing affinities. In particular, calsequestrin which is the second most abundant protein in the membranes, can bind 30-40 nmoles Ca^{2+}/mole with low affinity and it might play some role in Ca^{2+} storage.

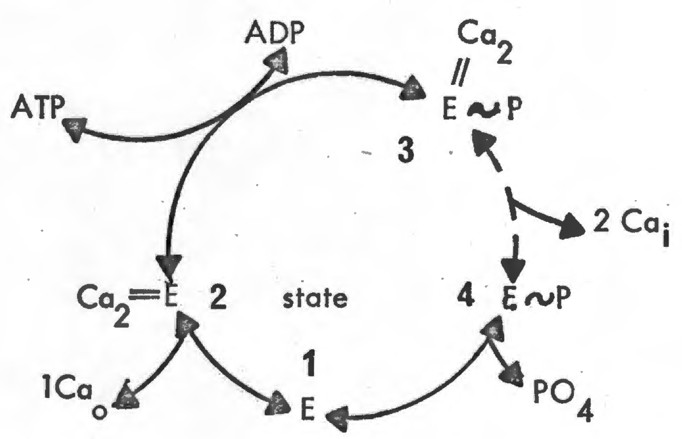
There is some doubt as to the form in which Ca^{2+} is stored. Early studies assumed that the Ca^{2+} accumulated was present as the free ion (Hasselbach, 1964). In the presence of oxalate there is no doubt but that the intravesicular concentration of Ca^{2+} exceeds the solubility product of calcium oxalate for precipitation of calcium oxalate to occur. Even so, Weber et al. (1966) have calculated that 30-50% of Ca^{2+} accumulated in the presence of oxalate is membrane-bound. Without oxalate at least 80% of the Ca^{2+} accumulated is membrane-bound (Carvalho, 1972; Ebashi and Endo, 1968) and the Ca^{2+} released into the sarcoplasm to initiate contraction must, to a large extent, come from this source.

Three types of processes for Ca^{2+} release are known to occur in the FSR. A slow passive efflux can be demonstrated if the FSR vesicles are allowed to accumulate Ca^{2+} maximally in the presence of oxalate and the uptake process then inhibited by adding EDTA to chelate the Mg^{2+} ions or by exhaustion of ATP supply (Johnson and Inesi, 1969) Efflux can be activated in the presence of high ADP and Pi concentrations once the vesicles have accumulated maximally (Barlogie et al., 1971; Makinose, 1971). Both these processes are too slow to account for contraction in muscle. A third and faster release was found by Entman et al., (1972) using canine heart SR. A spontaneous release of Ca^{2+} was observed to follow Ca-Binding. The physiological significance of this finding, however, is unknown. Activation of contraction is thought to occur through a release of Ca^{2+} into the sarcoplasm through depolarisation of the reticular membrane. The release mechanisms described above, although perhaps not responsible for initiation of contraction, possibly do occur in vivo and contribute towards the steady-state rate of ATP hydrolysis.

Since ATP-ADP exchange (Ulbrecht, 1962) accompanies the hydrolysis of ATP, a phosphorylated intermediate could be involved in both ATPase activity and Ca^{2+} accumulation (Hasselbach and Makinose, 1962). There is now ample evidence for the phosphorylation of FSR membranes by ATP-^{32}P (Yamamoto and Tonomura 1967, 1968; Martonosi 1967, 1969). It has an absolute requirement for Ca^{2+} and the concentration range of Ca^{2+} that is required for optimal formation is the same as that required for optimal ATPase activity.

Hydrolysis of the phosphorylated intermediate proceeds at rates consistent with those of the ATPase-mediated hydrolysis of ATP, indicating that hydrolysis rather than phosphoryl transfer is the rate-limiting step. Meissner et al., 1973, have shown that partially purified ATPase protein has two Ca^{2+}-binding sites and one ATP-binding site per phosphorylated site.

A number of reaction mechanisms have been proposed in recent years, to describe the binding of Ca^{2+} and of ATP to the enzyme, formation of a covalently-linked phosphoprotein intermediate, Ca^{2+} translocation and finally the breakdown of the phosphorylated intermediate (Kanazawa et al., 1971; Makinose, 1969, 1973; Meissner, 1973). The whole process is reversible and ATP synthesis can be coupled to Ca^{2+} efflux (Makinose and Hasselbach, 1971; Barlogie et al., 1971; Makinose, 1971). A scheme for the mechanism of Ca^{2+} transport proposed by Makinose (1973) is as follows,



In the forward reaction, when Ca^{2+} is accumulated, the enzyme binds two calcium ions on the external surface of the vesicular membrane (State 1–2).

It reacts with NTP and forms phosphoprotein (state 2-3). The phosphorylated intermediate releases Ca^{2+} into the interior of the vesicles (state 3-4) and subsequently is dephosphorylated (state 4-1). State 1 and 2 takes place on the external surface and state 4 on the internal surface of the vesicular membrane. The localization of state 3 remains to be defined .

1.6 LIPID REQUIREMENTS FOR ATPASE ACTIVITY AND CA^{2+} TRANSPORT BY SARCOPLASMIC RETICULUM.

Lipids are essential for the activation of Ca^{2+}-stimulated ATPase, and hence for Ca^{2+} transport, but the lipid requirements appear to be different for each process. In addition, formation of the phosphoprotein intermediate has specific lipid requirements. Treatment of FSR membranes with unsaturated fatty acids (Hasselbach and Makinose, 1962), mild phospholipase A (Fiehn and Hasselbach, 1970) or aqueous diethyl ether (Inesi _et al._, 1967) prevents Ca^{2+} accumulation without inhibiting the Mg^{2+} - and Ca^{2+} - stimulated ATPase activity. A possible explanation is that each of these processes renders the membrane more permeable, resulting in the inability of the vesicles to maintain a concentration gradient. ATPase activity is often greater in these treated vesicles.

After digestion with phospholipase A, the fission products remain firmly bound to the membrane. Removal on bovine serum albumin abolishes ATPase activity and prevents formation of the phosphoprotein intermediate. Addition of unsaturated fatty acids, with chain length 16-18 and double bonds in the middle of the chain, particularly oleate, restores both processes. Lysophosphatidyl choline reactivates only the ATPase activity. The Ca^{2+}-accumulating ability cannot be restored (Fiehn and Hasselbach, 1970; The and Hasselbach, 1973). ATPase activity and Ca^{2+} accumulation can also be dissociated from one another by means of a series of nonionic and anionic detergents which activate ATPase activity but not Ca^{2+} accumulation. Surprisingly, the degree of unsaturation of the fatty acid side chains of the phospholipids was not important for either process.

Treatment with phospholipase C has established the essential nature of the link between the polar choline phosphate and the hydrophobic acyl chains since lipase-treated FSR loses both ATPase activity and the ability to transport Ca^{2+} (Martonosi *et al.*, 1968).

According to Fiehn and Migala, 1971, phospholipase action does not alter the affinity of the SR membranes for Ca^{2+}. Carvalho (1972), however, found that digestion with lipase diminished the number of Ca^{2+}-specific binding sites but enlarged the total cation binding capacity. Martonosi has produced evidence that the inhibition of ATP hydrolysis might primarily be due to inhibition of the decomposition of the phosphoprotein intermediate (Martonosi *et al.*, 1971).

Purified ATPase preparations can form vesicles, with exogenous phospholipids, which are capable of accumulating Ca^{2+} (Meissner and Fleischer, 1974; Racker, 1972). This promises to be an effective way of establishing the exact lipid requirements of both functional processes. Results reveal that the most effective combination of phospholipids is that present in FSR membranes. Nevertheless, the reconstituted vesicles most active in Ca^{2+} accumulation only have 25% of the concentrating ability of controls and have an elevated ATPase activity similar to preparations obtained with diethyl ether treatment, and an increased permeability of the membrane to calcium is envisaged. The role of the extrinsic proteins could, therefore, be to decrease permeability of the membrane.

Purified ATPase or FSR preparations are able to be solubilised by deoxycholeate. Removal of the detergent leads to spontaneous formation of vesicles that are capable of accumulating Ca^{2+}. (Meissner and Fleischer, 1974). A technique, developed by Metcalfe and coworkers (Warren et al., 1974), in which purified ATPase is solubilised with detergent in the presence of excess exogenous lipid eg. dioleoyl phosphatidyl choline, and centrifuged in a discontinuous sucrose gradient, permitting exchange of endogenous with exogenous lipids, also promises to be a useful method for controlling the lipid environment of the ATPase enzyme.

Large differences in the cholesterol content and phospholipid composition of FSR isolated from chickens with hereditary dystrophies have been reported (Owens and Hughes, 1970; Hsu and Kaldor, 1971). These diseases exhibit decreased Ca^{2+} accumulation in the FSR whilst ATPase activity remains unchanged; the alterations in lipid could be responsible.

Boland et al., (1974) have examined the profiles of lipid and protein on attainment of Ca^{2+}-stimulated ATPase activity and Ca^{2+}-accumulating ability during development. These authors, using FSR isolated from skeletal muscle of chick embryos, showed that the rapid rise in rate and extent of Ca^{2+} accumulation around the time of hatching was accompanied by increased Ca^{2+}-stimulated ATPase activity, increased concentration of phosphoprotein intermediate and quantity of ATPase enzyme. Although the relative amounts of phospholipids in the membrane changed little during development, the appearance of activity in Ca^{2+} transport was accompanied by a marked decline in the palmitate, and rise in the linoleate content of membrane phospholipids and a fall in the cholesterol content.

Seiler and coworkers reported that administration of 20, 25-diazocholesterol to rats lowered the proportion of the fatty acids of the $\omega 9$ series in skeletal muscle FSR (Seiler and Kuhn, 1971) whilst feeding a diet free of essential fatty acids had the opposite effect (Seiler and Hasselbach, 1971). In both instances, however, initial rate and total amount of Ca^{2+} taken up by the reticulum were increased, the 'basal' ATPase unaffected and the 'extra' ATPase decreased. They explained their results in terms of a decreased permeability to Ca^{2+}, which was borne out by a decreased Ca^{2+} efflux rate from the vesicles of treated rats. However, Masoro andYu (1971), feeding rats on a diet free of essential fatty acids and Tume <u>et al</u>., (1973) supplementing the diet with sunflower oil or hydrogenated coconut oil found similar changes in the fatty acid profiles to those noted by Seiler and coworkers but were unable to detect changes in the activity of the SR pump. Significant changes in the degree of unsaturation in the fatty acids of the membrane were not observed.

Although these observations do not establish a causal relationship, they do emphasise the importance of hydrophobic interactions in the proper functioning of the membrane. Changes in the fatty acid side chains of phospholipids, have been correlated with the binding of Ca^{2+} and the ionic structure of phosphatidyl choline monolayers (Shah, 1970). The preponderance of fatty acids with a chain length between 16 and 18 as the most effective activators of membranes that have been treated with phospholipase C and then albumin, is consistent with their optimum hydrophobic character as defined by Mukerjee (1965). In spite of the fact that in reconstituted preparations lipids with differing charge are present, their activity patterns are similar with respect to the dependence on Ca^{2+}, $Mg.ATP^{2-}$, and pH suggesting that lipids may play a structural role rather than exert direct influence on the active site.

6.0 MATERIALS AND METHODS.

2.1 PREPARATIVE PROCEDURES.

2.1.1 SELECTION OF SKELETAL MUSCLE SAMPLES.

The muscle used throughout this investigation was sampled from adult Landrace x Large White cross pigs, which had been fed on different pig feeds depending on the farm of origin and weighed 90–100 kg at the time of slaughter. The pigs were transported from surrounding farms in open trucks, and were penned at the Spekenam Abattoirs, Kuilsrivier, Cape, for 12–24 hours with free access to water.

The animals were led singly into the slaughter room and slaughtered in groups of 4 or 5 by application of 80 volts of alternating current (50 c.p.s.) *via* electrodes pressed to the head for approximately 10 sec. The stunned animals were hoisted by means of their hind legs and exsanguinated 15–30 sec after stunning by severing the right jugular vein. The carcasses were immersed in a water bath (approx. 50°C) and fired in a butane gas burner for 5–10 sec in order to remove body hair.

Muscle samples were removed within 10–15 min of stunning. The pH of the muscle *in situ* was measured by means of a combined glass-calomel electrode and a type 29 portable pH meter (Radiometer, Denmark). The electrode was calibrated against pH 7,00 standard buffer (Beckman Instruments (PTY) LTD. Cape Town) at room temperature (18–22°C). An incision was made at the level of the twelfth thoracic vertebra (T12) and the pH electrode inserted 2–3 cm into the *longissimus dorsi* muscle. An average of 3 or 4 readings was taken. The muscle was also examined for colour, consistency and texture. The muscle was judged to be either slowly glycolysing (normal) or rapidly glycolysing (PSE) on the basis of colour, consistency, amount of exudation and pH (Table 1) Normal samples were selected from muscles which were red in colour, firm in consistency, which had no obvious watery exudate and which had a pH at 10–15 min *post-mortem* of 6,50–6,80. Samples of rapidly glycolysing muscle were taken from skeletal muscle which was obviously pale and soft, which exuded a watery fluid and which had a pH 10–15 min *post-mortem* of 5,40–5,70. The quantity of muscle excised depended on the investigations to be performed.

The rate of cooling of muscle samples during transportation on ice to the laboratory was considered. In several experiments the temperature of the muscle sample was continuously monitored by means of a thermistor probe (Yellow Springs Instrument Co. Inc. Yellow Springs, Ohio) inserted into the centre of the muscle sample. The rate of cooling depended on the mass of the sample. A 200 g sample of muscle which was 39°C on excision, cooled at an approximately linear rate to 7°C on arrival at the laboratory after 35 min in transit. The change in pH of samples during transit was also monitored. The pH of PSE muscle decreased approximately 0,1 pH units and slowly glycolysing muscle 0,4 pH units during the same period.

2.1.2 EXTRACTION OF TOTAL LIPIDS FROM SKELETAL MUSCLE AND FROM FRAGMENTED SARCOPLASMIC RETICULUM.

The chloroform-methanol method of Folch et al. (1957), in which the lipids and hydrophilic components are partitioned into organic and aqueous phases respectively, was employed.

Procedures were conducted at room temperature.

(i) Whole Muscle.

Freshly excised tissue (1g) cut into 0,4 cm cubes was homogenised by means of an Ultra Turrax homogeniser (Janke and Kunkel KG., Strauken i. Br.) by 3 bursts each of 5 sec duration at half maximal speed in 17 vols chloroform-methanol, 2:1. A further 3 vols of chloroform-methanol, 2:1, were used to rinse the homogeniser probe and added to the homogenate. After filtration through a filter paper that had been rendered fat free by a previous chloroform-methanol, 2:1 extraction, the filtrate was washed with 0,2 vols of 0,5% aqueous NaCl. NaCl was included in the wash to help prevent loss of acidic phospholipids into the aqueous phase (Folch et al., 1957). After siphoning off the upper aqueous phase, the lipid extract was washed thrice with 'pure solvents upper phase' (chloroform-methanol-water, 3:48:47) and converted into a single phase with a minimum of methanol.

Butylated hydroxytoluene (BHT) (Sigma, St Louis, USA) was added as an antioxidant to a concentration of 0,005%.

(ii) FSR.

FSR suspension (1 ml of approx. 2 mg FSR protein/ml) was homogenised in chloroform-methanol, 2:1 (10 ml), by 3 bursts each of 45 sec duration at about $^1/4$ maximal speed with a MSE 77030 homogeniser equipped with 1,1 cm diam. blades. Aggregated material was dislodged from the blades with a micro spatula and the mixture homogenised with additional solvent mixture (5 ml) for a single burst of 1 min duration at the same speed.

The homogenate was then filtered, by means of a millipore apparatus (Millipore Corp. USA), through a Whatman No.1 filter paper in millilitre amounts. Solvent mixture (3 ml) was used to rinse the probe, homogenising container (20 ml scintillation counting vial; Packard Instruments, Zurich, Switzerland), and residue plus filter.

The subsequent procedure was exactly as described for the extraction of whole muscle.

2.1.3 SEPARATION OF TOTAL LIPIDS INTO 'NEUTRAL' AND 'POLAR' FRACTIONS BY MEANS OF SILICIC ACID COLUMN CHROMATOGRAPHY.

The total lipid extracts from whole muscle and FSR suspensions were separated into 'neutral' and 'polar' fractions by means of silicic acid chromatography according to the method of Dittmer and Wells (1969). 'Neutral' moeities do not bind to silicic acid in chloroform and are eluted with this solvent. 'Polar' lipids can be subsequently eluted with methanol.

The fine particles were removed from the silicic acid (chromatography grade; BDH, Poole, England) to increase the flow rate. This was accomplished by suspending silicic acid (5 g) in methanol (100 ml) in a measuring cylinder and allowing it to settle. The supernatant was decanted after the majority of the acid had settled. This procedure was repeated three times.

The silicic acid was then oven dried (110°C) for 1 hour. A slurry of adsorbent in chloroform was poured into a glass column (0,9cm int. diam.) with stopcock and pyrex glass wool to retain the particles, to give a column bed height of 1,5 cm. The column was washed with chloroform (5 ml). The 'neutral' lipids were eluted with chloroform (20 ml) at a flow rate of approximately 0,5 ml/min. The 'polar' lipids were subsequently eluted with methanol (20 ml).

The quantity of lipid which had been applied to the column and the recovery was determined by microweighing aliquots on a Cahn microbalance (see page 76). Recoveries ranged from 65 to 80%.

2.1.4 ISOLATION AND PURIFICATION OF FRAGMENTED SARCOPLASMIC RETICULUM (FSR).

During differential centrifugation fractionation procedures, FSR sediments with the microsomal fraction and is usually contaminated by actomyosin and fragments of other subcellular particles. Actomyosin can largely be eliminated by dissolution at high ionic strength (Uchida et al., 1965). Further purification of the crude fraction can be achieved by bouyant density centrifugation in sucrose gradients.

The method described was adapted from that of Greaser et al., (1969d).

(i) Differential Centrifugation.

(All operations were performed at 2-4°C).

Minced skeletal muscle (50-200 g) was homogenized in 4 vols ice-cold 0,1 M KCl, 5 mM histidine, pH 7,2, with a Waring Blender (Braun A.G. Frankfurt/M West Germany) for 30 sec at full speed. The homogenate was centrifuged at 1000 xg for 20 min and the pellet discarded. The supernatant was filtered through 4 layers of cheese cloth and solid KCl stirred in to a final molarity of 0,6 M to dissolve contaminating actomyosin (Owens et al., 1973).

The filtrate was centrifuged at 8 000 xg for 20 min and the pellet again discarded. The supernatant was then centrifuged at 30 000 xg (Beckman model G Ultracentrifuge, Beckman rotor 21) for 60 min. The sediment of crude FSR was gently suspended in 0,1 M KCl, 5 mM histidine, pH 7,2, to a concentration of approximately 25 mg protein/ml, using a small Potter-Elvehjem (teflon-glass) homogeniser.

(ii) Discontinuous Sucrose Density Gradient Centrifugation.

The membrane suspensions (approx. 2 ml containing 50 mg of crude FSR protein) were layered on top of a discontinuous sucrose density gradient prepared with 35%, 40% and 45% ($^w/v$) sucrose (8 ml each) at 2-4°C, and centrifuged in a swinging bucket rotor (Beckman SW-25.1 rotor) at 64 700 xg (25 000 rpm) for 2 hours. The crude FSR from slowly glycolysing (normal) muscle banded in 3 distinct regions, namely at the 40-45% sucrose interface, the 35-40% sucrose interface and on the surface of the 35% sucrose layer (fig 10). These bands were designated N_1, N_2 and N_3 respectively. The microsomal fraction from PSE muscle only banded at the 35-40% sucrose interface and on the surface of the 35% sucrose layer. These were designated P_2 and P_3 respectively. The region equivalent to N_1, namely P_1, contained no visible material (see Results). Separation of the layers was accomplished by eluting through a puncture hole at the bottom of the tube. The suspensions obtained from the gradient were diluted with 0,1 M KCl, 5 mM histidine, pH 7,2, to a volume of 36 ml and sedimented by centrifugation at 60 000 xg for 30 min (Beckman rotor 30). The pellets of the enriched fragments were resuspended in 0,1 M KCl, 5 mM histidine, pH7,2, and stored at 0-3°C.

(iii) Continuous Sucrose Gradients.

The apparatus and procedure was described by Stead *et al*., (1964) who adapted the method of Britton and Roberts (1960).

The apparatus consisted of two glass receptacles containing equal volumes of sucrose; one 15% sucrose and the other 45% ($^{w}/v$) sucrose, connected at the bottom by a narrow bore tube. The tube containing the denser sucrose was mechanically stirred and an exit tube led from this reservoir to the gradient tube. Gradients were prepared at room temperature, cooled for 30 min in a cold room at 2-4°C and used immediately. The samples (2 ml of approx. 25 mg protein/ml) were applied and centrifuged in a swinging bucket rotor (Beckman SW - 27,1) for 16 hours at 80 000 xg (23 000 rpm). The crude material separated into 3 distinct populations of fragments, namely an aggregated band, a broad disperse band occupying the central region of the gradient and a narrow monodisperse band. The aggregated and monodisperse bands varied in bouyant density depending on whether the microsomal fraction originated from slowly glycolysing or PSE muscle (fig 15). The aggregated material from PSE muscle invariably contaminated the broad disperse band and the two could not be completely separated.

The contents of the gradient tubes were eluted through a puncture hole at the bottom of the tube either by air pressure in which case the three fractions were collected directly, diluted with buffer, the material pelleted and resuspended as described in (ii) above or by pumping in H_2O (Pump type 5, Distillers Comp., Surrey, England) from above at the rate of 0,8 ml/min and the effluent monitored for UV absorption at 280 nm by passage through a 2 mm flow cell in a Unicam SP 1800 spectrophotometer. The eluate was collected in approx 0,5 ml fractions.

Sucrose concentrations and their densities were determined by refractometry at room temperature using a refractometer (Bellingham and Stanley, London) in conjunction with a sodium lamp. The refractometer was set at 3,0000° with H_2O and the following equation, which was experimentally determined by linear regression analysis from data of standard sucrose solutions, was used for calculations

Density (at 0°C) = 0,03403 x + 0,89362

Where x = number of degrees (in decimals) of refraction for sodium light.

2.1.5 ACID TREATMENT OF HOMOGENATE FROM NORMAL MUSCLE.

All procedures were carried out at 2-4°C unless otherwise stated.

A quantity (50 g) of freshly excised slowly-glycolysing muscle was minced and dispersed in 0,1M KCl, 5 mM histidine buffer, pH 7,2 (50 ml). The pH of the mixture was adjusted to pH 5,6 with 90% L (+) - lactic acid (Fluka H G, Buchs S G, Switzerland) drop by drop with vigorous manual stirring. The acidified mixture was placed in a waterbath at 50°C and manually stirred. When the temperature of the mixture reached 35°C it was transferred to a waterbath at 37°C and maintained at this temperature for 10 min. Ice-cold buffer (150 ml) was added which brought the temperature to 9°C and the pH to 5,9. The pH was adjusted to pH 7,2. The subsequent homogenisation and FSR isolation was as described in section 2.1.4.

2.2 ANALYTICAL PROCEDURES.

2.2.1 DETERMINATION OF LACTATE IN MUSCLE SAMPLES.

The method used consisted of spectrophotometric measurement of the appearance of NADH during the enzymatic oxidation of lactate to pyruvate by LDH (Hohorst, 1965).

$$\text{L-(+)-Lactate} + NAD^{+} = \text{pyruvate} + NADH + H^{+}$$

The equilibrium of the reaction ($K_D = 2{,}9.10^{-12}$) lies far to the left and in order to obtain the quantitative oxidation of lactate the reaction products of protons and pyruvate are trapped by the use of an alkaline reaction medium and hydrazine respectively. The equation then becomes

$$\text{L-(+)-Lactate} + NAD^{+} + \text{hydrazine} \xrightarrow[\text{pH } 9{,}5]{\text{LDH}} \text{pyruvate hydrazone} + NADH + H_3O^{+}$$

with an equilibrium constant, K_D of $7{,}10^{2}$.

Freshly excised muscle (0,5-1,5 g) was placed in preweighed vials containing ice-cold 50% perchloric acid (PCA, 5 ml) and transported to the laboratory in ice. Within an hour of sampling the tissue was homogenised with an Ultra Turrax homogeniser (Janke and Kunkel K.G. Shauken i. Br.) in 3 bursts each of 7 sec duration. The homogenate was diluted with ice-cold 50% perchloric acid to give a ratio of volume of extract to tissue weight of 10:1. The suspension was thoroughly mixed and centrifuged at 7,000 rpm for 20 min at 4°C.

The assay medium (2,0 ml) contained 0,38 M hydrazine, 0,95 M glycine, pH 9,5, and 4,5 mM NAD.

The PCA extract (0,02 and 0,10 ml) was added and the reaction initiated by 50 µg LDH (Seravac Laboratories, Epping Industria, Cape Town). The reaction was monitored at 340 nm and shown to be 95% complete after 10-15 min. Final readings were made after 1 hour.

Calculation of lactate concentration was based upon the assay which gave A_{340} nearest to 0,4 absorbance units and upon millimolar extinction coefficient for NADH of 6,22.

Standards containing 1 mM L-(+)-Lactate (Fluka A.G., Buchs S.G., Switzerland) were determined with the samples. Recovery of a 1 mM standard solution L-(+)-Lactate ranged from 90-95%.

2.2.2 DETERMINATION OF GLYCOGEN IN MUSCLE SAMPLES.

The method Tarnocky and Nagy (1963) was followed. Glycogen is precipitated from a TCA extract of tissue with ethanol. The washed precipitate is hydrolysed in acid and the liberated glucose determined by the o-toluidine method.

Freshly excised muscle (0,5-1,5 g) was immediately placed in pre-weighed vials containing ice-cold 10% trichloracetic acid (5 ml) and transported to the laboratory on ice. Within an hour of sampling the tissue was homogenised with an Ultra Turrax homogeniser in 3 bursts at medium speed, each of 7 sec duration. The homogenate was diluted with ice-cold 10% TCA to give a ratio of volume of extract to tissue weight of 30:1. The suspension was thoroughly mixed and then centrifuged at 1000 xg for 10 min at 4°C.

Supernant (2 ml) was mixed with absolute ethanol (4 ml) and allowed to stand for 16 hours at 4°C. The mixture was centrifuged at 1 000 xg for 20 min at 4°C, the supernatant carefully decanted and the pellet dried by inverting the tube on tissue paper. The pellet was suspended in 2,0 N H_2SO_4 (0,5 ml) and dissolved by heating in a boiling water bath for 1 hour. After cooling 6% ($^w/v$) o-toluidine, 0,15% ($^w/v$) thiourea in glacial acetic acid (8,0 ml) was added, the contents of the tube mixed and the green colour developed for 8 min in a boiling water bath.

Standards containing up to 2,78 µmoles glucose were codetermined with the samples.

The absorption of the green colour was read at 635 nm and the glycogen content expressed as µmoles of glucose equivalents per g tissue.

2.2.3 DETERMINATION OF PHOSPHORUS IN LIPID EXTRACTS.

The method followed was that described by Kates (1972), which was based on the micro-procedure of Bartlett (1959) and consists of conversion of organic P to reduced phosphomolybdate.

Aliquots of lipid extract (0,5–10 µg P) were dried on the bottom of a 150 x 15 mm pyrex tube in a stream of N_2 gas. A solution of 72% perchloric acid (0,4 ml) was added and the tubes heated at an angle over an electric heater. Heating was adjusted by means of a 'Simmerstat' so that perchloric acid vapour refluxed approximately 3 cms from the tops of the tubes. Once the solutions were colourless, the tubes were allowed to cool and water (4,2 ml), 5% ammonium molybdate (0,2 ml) and 1% amidol (2,4 - diaminophenol - dihydrochloride) in 20% sodium bisulphite (0,2 ml) were added and the contents of the tubes well mixed. The tubes were heated in a boiling water bath for 7 min to develop the blue colour. After standing for 15 min the absorbance was read at 830 nm.

Standards (1,4 and 9 µg P) were co-determined with the samples.

2.2.4 DETERMINATION OF PROTEIN.

The Biuret (Gornall et al., 1949) and Lowry (Lowry et al., 1951) methods were used to determine protein concentrations of solutions and particulate suspensions. The Biuret method was preferred at higher (10 mg protein/ml) and the Lowry method at lower protein concentrations.

(i) Biuret Method.

The samples (0,05 ml containing 0,2 to 1,0 mg protein) was mixed with 10% sodium deoxycholate solution (0,01 ml) and 0,1 N NaOH (0,2 ml) was added. Biuret reagent (1 ml) was added with mixing and the solution allowed to stand for 30 min before the absorbance was read at 540 nm.

Standards of bovine serum albumin (10 mg/ml) were determined with each batch of samples.

(ii) Lowry's Method.

The protein sample (0,005 - 0,02 ml containing 20 to 100 µg protein) was shaken with 10% sodium deoxycholate (0,05 ml) and the volume made up to 2 ml with H_2O. Alkaline copper solution (1 ml) was added and the mixture allowed to stand for 10 min. Folin-Ciocalteau reagent (0,10 ml) was added with vigorous mixing and after 30 min development, the colour was read at 750 nm.

Standards of 25, 50, and 100 µg bovine serum albumin (Sigma, St Louis, USA) were determined with each batch of samples. The protein concentration was calculated graphically from the standard curve.

All protein determinations were performed in duplicate.

2.2.5 DETERMINATION OF CHOLESTEROL IN LIPID EXTRACTS OF FRAGMENTED SARCOPLASMIC RETICULUM.

The method described by Kates (1972), adapted from that of Courchaine *et al*., (1959), was followed.

An aliquot of chloroform-methanol (2:1) extract of lipids (0,5 ml) containing up to 0,06 mg total cholesterol was placed in a 12,5 x 1,3 mm test tube and evaporated to dryness in a stream of nitrogen at room temperature. Glacial acetic acid (1,2 ml) was added, the solution mixed, dilute ferric chloride solution (0,8 ml) was added and again mixed. The contents were allowed to cool and the absorbance of the purple colour read at 550 nm after 10 min development.

A standard curve was prepared from standard solution of cholesterol (BDH, Poole, England) in isopropanol containing 0,01, 0,04 and 0,06 mg cholesterol per tube.

All determinations were performed in duplicate.

2.2.6 THIN LAYER CHROMATOGRAPHY OF 'NEUTRAL' LIPIDS. AND PHOSPHOLIPIDS.

Thin layer chromatography of lipid extracts on thin layers of silica gel was performed as described by Skipski and Barclay (1969).

(i) Preparation of Chromatoplates.

Glass plates (200 x 200 x 3 mm) were washed with tap water, rinsed several times in distilled water, followed by absolute ethanol and then allowed to dry.

Two different adsorbants were used. Silica gel G (E. Merck, Darmstadt, Germany) was used for chromatographing the 'neutral' and 'polar' lipids of whole muscle extracts and the neutral lipids of extracts of FSR. Silica gel H (Merck, Darmstadt, Germany) made into a slurry with aqueous 1 mM Na_2CO_3, was used to separate the phospholipids extracted from FSR. A slurry of either adsorbant was spread on the plates with an applicator (Quickfit Instruments, England) to a thickness of 0,5 mm. The chromatoplates were dried at room temperature and then stored. Before an experiment the plates were activated in an oven (120°C) for 1 hour. They were cooled in an applicator or spotting box (fig 3) and the sample to be chromatographed was applied within a few minutes. The box was flushed with dry N_2 during the cooling and during the application of the sample.

This standardised procedure resulted in greater reproducibility of Rf values of lipid classes than was otherwise obtainable.

(ii) Application of the Sample.

Extracts (approx. 0,5 ml total lipid) were dried in a stream of N_2 at room temperature and the lipids redissolved in chloroform (approx 0,02 ml). The sample was spotted in a 2 cm band 3,0 cm from the bottom edge of the plate with a 50 µl Hamilton syringe.

Standard phospholipid (approx. 50 µg) or neutral lipid mixture was applied to every plate alongside the sample (see fig 17).

(iii) Development of Chromatograms.

The chromatographic chamber was lined on 2 sides with filter paper (Whatmans No. 1) and allowed to equilibrate with developing solvent (200 ml) for 1 hour.

Three different developing solvents were used. The neutral lipids were chromatographed using silica gel G and the solvent system of Mangold *et al.* (1960) (Solvent system I) which consists of diethylether, acetic acid and petroleum ether (b.p. 60°-80°C) 90: 10: 1.

Fig. 3 Spotting box used in thin-layer chromatography.

The box was constantly flushed with dry nitrogen during the application of the lipid samples. Greater reproducibility in lipid separation patterns were obtained if the chromatoplate was cooled in the box after activation and the samples applied once the plate reached room temperature.

The phospholipids from whole muscle extracts were developed on silica gel G with chloroform, methanol (4:1) containing 2% pyridine and 2% H_2O, as described by Dittmer and Lester (1964) (solvent system II). The phospholipids extracted from FSR were separated on basic silica gel G with the solvent system recommended by Skipski and Barclay (1969) consisting of chloroform, methanol, acetic acid and water (25:15:4:2) (solvent system III). Solid butylated hydroxy-toluene (Sigma, St Louis, USA) was dissolved in the solvents to a final concentration of 0,005% to act as an antioxidant.

The chromatograms were allowed to run until the solvent front was within 4 cm of the top of the plate (approx. 1½ hours).

(iv) Detection.

If the lipids were to be analysed by GLC the plate was sprayed with 0,05% aqueous Rhodomine 6G (BDH, Poole, England) and the spots viewed under UV light.

For photographic purposes the plates were sprayed with 50% aqueous H_2SO_4 and heated on a copper plate over an electric element. The charred spots appeared within 2-4 min.

Standards were run alongside samples to aid identification. Standards of LPCh, Sph (Sigma, St Louis, USA) PCh (Sigma), PE and cholesterol, free fatty acids (Stearate, Oleate), triolein, methyloleate, cholesteryl oleate were used (Applied Science Laboratories, Pennsylvania, USA, mixture TLC - 1 and TLC - 3). PS was identified by staining with 0,2% ninhydrin in butanol saturated with water. After spraying, the chromatogram was heated for approximately 5 min on a copper plate over an electric heating element while a stream of steam was directed onto the silica gel. PE and PS showed up as red-violet spots.

(v) Recovery of Lipids Separated by TLC.

The spots corresponding to the various lipid classes were removed by moistening the area with a drop of H_2O and carefully lifting the spot with a razor blade.

(vi) Quantitative Analysis of Separated Phospholipid Classes.

The procedure adopted was that described by Skipsi and Barclay (1969).

A measured quantity of total phospholipid mixture (25-30 μg P) was applied to a chromatoplate. The chromatogram was developed in chloroform, methanol, acetic acid and water (25:15:4:2) and an area of silica gel corresponding to various phospholipid spots was quantitatively transferred to a conical centrifuge tube. Phospholipid was eluted from the silica gel powder by successive vigorous shaking in 3 and 2 mls of chloroform, methanol, acetic acid and water (25:15:4:2), 2 ml methanol, and 2 ml methanol, acetic acid and water (95:1:5).

The extracts were combined in graduated 12 ml tubes and the volume made up to 12 ml with chloroform. An aliquot of 8 ml was taken for methylation for subsequent GLC analysis. The remaining 4 ml was evaporated to dryness with a stream of N_2 and total P determined on the residue (see page 64).

Recoveries of lipid P were calculated and ranged from 75 to 98% with a mean of 86% and S.D. of 9,4% for 14 determinations.

2.2.7 GAS LIQUID CHROMATOGRAPHY OF FATTY ACIDS.

(i) Fatty Acid Methyl Esters.

Fatty acid methyl esters were prepared by the BF_3 method as described by Ackman (1969).

The lipid (up to 200 μg lipid P) was dried thoroughly at room temperature in a stream of nitrogen. Boron trifluoride, 14% in methanol, (1 ml) (BDH, Poole, England) was added and the mixture refluxed in a boiling water bath for 15 min. In later experiments an adaptation of the method of Dittmer and Wells (1969) was used and methylation was performed in sealed tubes. After adding the lipid sample and BF_3 methylating solution, the tubes were flushed with a stream of N_2 before sealing and incubated at 70°C for 15 min.

After cooling, distilled water (2 ml) and n-heptane (2 ml) were added and thoroughly mixed. On standing two layers separated. The top organic layer was removed and retained. The aqueous layer was washed twice with 0,5 ml n-heptane and the organic phases added to the first. The final volume of the extract was reduced in volume by evaporation in a stream of N_2, so that the lipid concentration was approximately 200 μg lipid/ml.

Excess moisture was removed with a small quantity of anydrous sodium sulphate which was added to the fatty acid methyl ester solution.

(ii) Preparations of Columns for GLC.

Column packing material was either obtained precoated from Applied Science Laboratories (Pennsylvania, USA) or prepared according to the method of Kates (1972).

Stationary phase (1-2 gm) was dissolved in warm chloroform (60 ml) in a round bottomed flask. Chromosorb W (10 g , Applied Science Laboratories) was slowly added with gentle swirling and the solvent removed on a rotary evaporator (Büchi, Switzerland) until a free-flowing granular material was obtained.

Two types of stationary phase were tried, diethylene glycol succinate (DEGS) and diethylene glycol adipate (DEGA)(Applied Science Laboratories). Both achieved good separation of the methyl esters, notibly C18:0 from C18:1, but the latter phase was generally used because of its increased stability in the working temperature range and hence longer life.

The columns (7 ft x 0,2 inch) were packed by plugging one end with glass wool and introduction of the packing at the other. The packing was shifted along the coiled glass column by judicial use of a hand vibrator and vacuum.

(iii) GLC of Fatty Acid Methyl Esters.

A Pye gas chromatograph (104 series) equipped with dual flame ionisation detectors was employed. The injection point temperature was 265^{0}C and the detector temperature kept at 270^{o}C. Satisfactory separations were achieved at column temperatures from 180–195^{o}C and a constant temperature of 188^{o}C was selected. N_2 was used as carrier gas at a flow rate of 30–40 ml/min.

Samples (1–25 μl) were injected onto the column by means of a Hamilton syringe.

Elution of the slowest migrating methyl ester (C24:2) was complete after 80–90 min. Attenuation of the ionisation current amplifier was usually set at 500 for the beginning of the run and decreased by a factor of 2,5 after C16:1 and 2 after C18:2 (fig 4).

The identification of individual fatty acid methyl esters was based on a comparison of the relative retention times of the sample peaks and those of standard mixtures under similar conditions. For this purpose a standard solution containing methyl esters of C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C20:3, C20:4, C20:5, C22:0, C22:1 and C24:1 was made up (Applied Science Laboratories). Peaks which did not correspond to any of the standards were tentatively identified from a graph of relative retention times to C18:0 versus chain length of the fatty acids with the same number of double bonds on semilogarithmic graph paper (fig 5).

Confirmation of the identity of individual components of selected unknown mixtures of fatty acid methyl esters was achieved by bromination and hydrogenation (see below). After bromination, chromatographs showed only peaks due to fully saturated (Cn:0) methyl esters. Hydrogenation of unsaturated fatty acids resulted in their elution along with the saturated ester series. These two chemical modifications were not performed routinely.

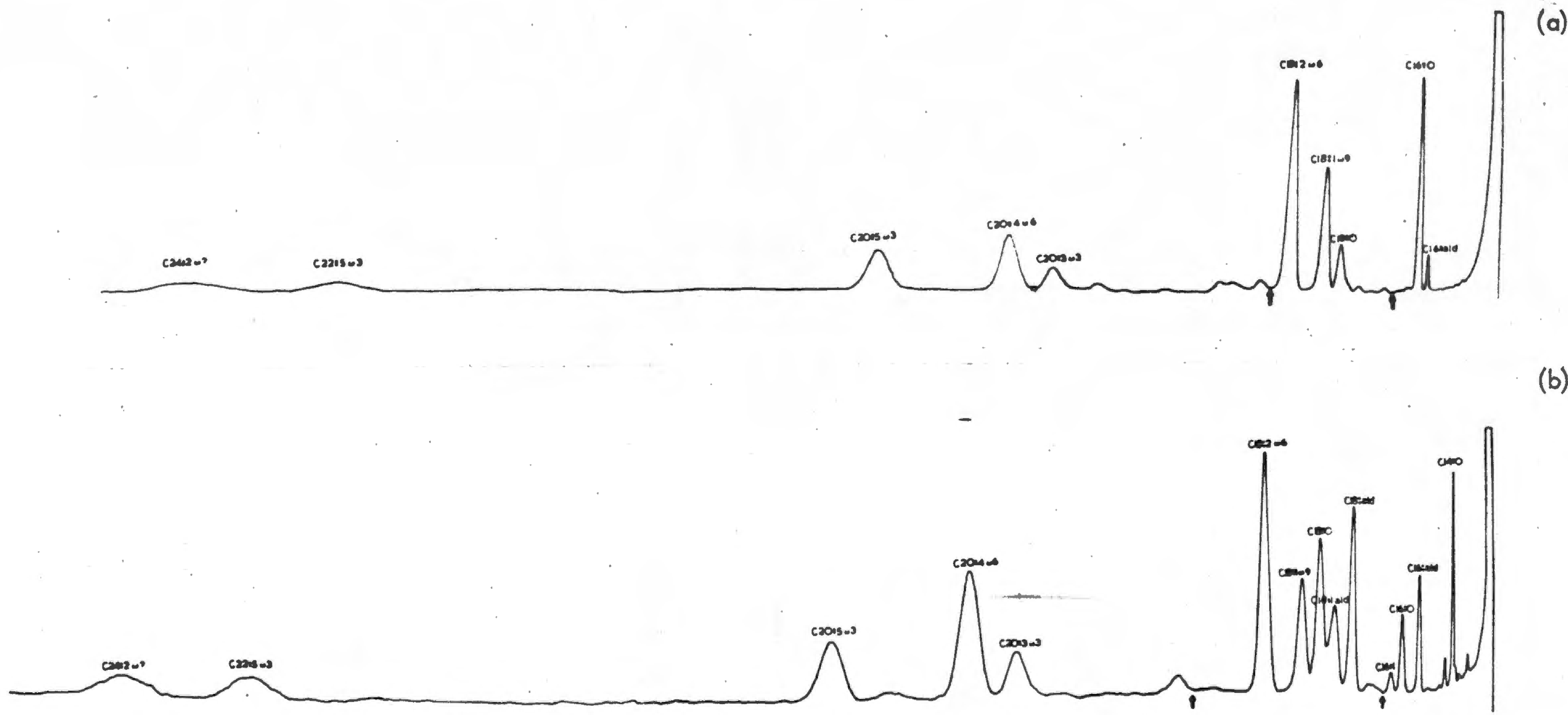


Fig. 4 Elution pattern of fatty acid methyl esters separated by gas-liquid chromatography.

The fatty acid methyl esters from phosphatidyl choline (a) and phosphatidyl ethanolamine (b) were separated on 10% DEGA in Chromosorb W at 188°C in a column 7 ft x 0,20 inches and with a N_2 flow rate of 35 ml/min. The entire run was approximately 90 min. The arrows indicate a decrease in attenuation first by a factor of 2,5 after the elution of palmitoleate and then 2 after linoleate

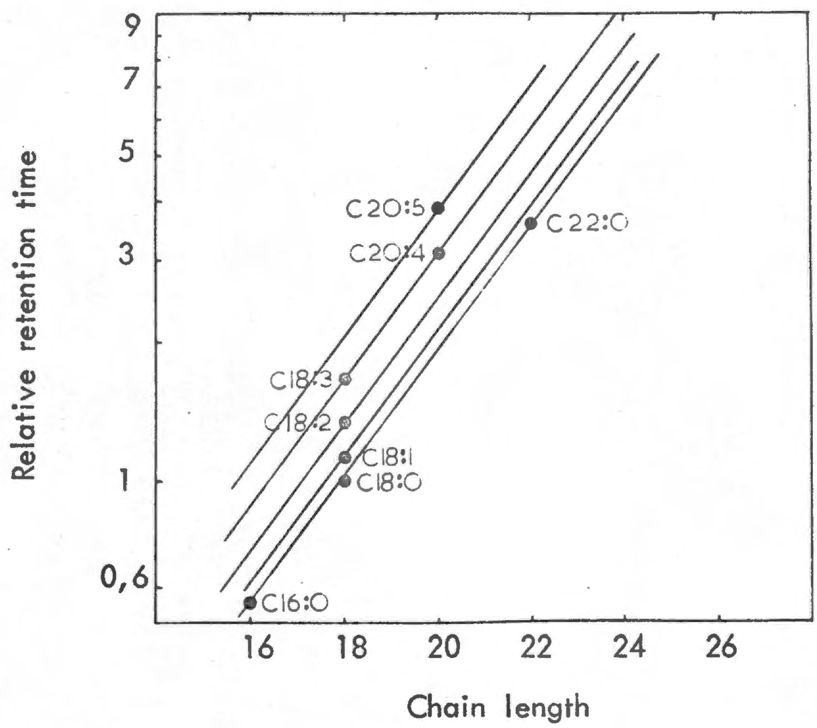


Fig. 5 Elucidation of the structure of fatty acids from gas-liquid chromatography data.

The dimethyl acetals were identified on the basis of published retention times (Farquhar *et al.*, 1959) and of results published by other workers (Marai and Kuksis, 1973; Joffe, 1969) which reveal that the PE class of phospholipid contains a relatively large number of vinyl ether linkages. The appearance of relatively large peaks with retention times expected of dimethyl acetals in the PE fraction led to the conclusion that these peaks represented methylation products of fatty aldehydes.

Typical chromatograms of methyl esters of fatty acids from PCh and PE are reproduced in fig 4.

(iv) Bromination of Fatty Acid Methyl Esters.

The method is that of Farquhar *et al* (1959).

A mixture of dried fatty acid methyl esters (approx. 50 µg) was dissolved in diethyl ether (1 ml) in a conical centrifuge tube and chilled to approximately -20°C in a beaker of dry ice in ethanol. A solution of bromine in diethyl ether (22%, $^{w}/v$) was added to the lipid mixture by means of a Hamilton syringe (50 µl) until the yellow colour persisted, indicating that bromination was complete. Excess bromine was evaporated with a stream of nitrogen whilst maintaining the volume at between 0,4 ml and 0,9 ml with diethyl ether until the solution became colourless. A sample was taken from the supernatant for GLC analysis.

(v) Hydrogenation of Unsaturated Fatty Acid Methyl Esters.

The method is that described by Farquhar *et al.*, (1959).

A mixture of fatty acid methyl esters (approx. 100 µg) was dissolved in ethanol (2 ml), platinum oxide (5 mg) was added and hydrogen was bubbled through the mixture at atmospheric pressure for 30 min. The suspension was centrifuged at 1 000 x g for 10 min. The supernatant was evaporated to dryness under N_2 and dissolved in n-heptane for subsequent GLC analysis. The whole procedure was performed at room temperature.

2.2.8 DODECYL SULPHATE POLYACRYAMIDE GEL ELECTROPHORESIS.

The method of Weber and Osborne (1969) was followed.

Protein (0,2-0,6 mg) was incubated for 2 hours at 37°C in 0,01 M Sodium phosphate buffer, pH 7,0 (1 ml) containing 1% dodecyl sulphate (sodium salt, SDS; BDH, Poole, England) and 1% β-mercaptoethanol. The solution was dialysed overnight at room temperature against 0,01 M Sodium phosphate buffer, pH 7,0 (500 ml), containing 0,1% SDS and 0,1% β-mercaptoethanol.

Electrophoresis was performed in glass tubes (75 x 5 mm). Before use they were rinsed in distilled water, then in 'Photo-Flo' (Eastman Kodak, New York) solution (1:200 dilution) and finally oven dried (110°C).

The gels were made in the following manner:
Gel buffer (0,029 M $NaH_2PO_4 . H_2O$, 0,072 M Na_2HPO_4 and 0,2% SDS (15 ml) was deaerated and added to ~~10%~~ 22,2% acrylamide (13,5 ml). After further deaeration, freshly prepared ammonium persulphate solution (1,5 ml, 15 mg/ml) and N,N,N - tetramethylethylenediamine (0,045 ml) were added. The solutions were mixed, and pipetted into the glass tubes to within 1 cm of the top. Before gelling occured a few drops of water were layered on the surface. Gelling occured within a few minutes. The water layer was poured off just before use and the tubes were placed in a Shandon electrophoresis apparatus (Shandon Scientific Co. London, N.W. 10). The sample was placed on the surface of the gel and the tube filled to the top with gel buffer which had been diluted 1:1 with distilled water. Each sample comtained tracking dye (3 µl 0,05% Bromophenol blue in water), glycerol (1 drop), β-mercaptoethanol (5 µl), dialysis buffer (50 µl) and protein solution (10-15 µl) containing 0,01-0,05 mg protein. The two compartments of the electrophoresis apparatus were filled with the gel buffer, diluted 1:1 with water. Electrophoresis was performed at a constant current (Spinco Duostat) of 8 ma per tube with the positive electrode in the lower chamber. At this current density, the voltage across the electrodes was approx. 30 volts. The marker dye moved $^3/4$ way down the gel in approx. 3 hours. The length of the gel and the distance moved by the dye were measured.

The gels were expelled from the tubes by introducing water from a syringe, between the gel and glass wall and applying pressure on one end. Staining was accomplished with Coomassie brilliant blue (1,25 gm dye in 454 ml 50% methanol, 46 ml acetic acid) in 5 hours and destaining by first soaking the gels in a destaining solution (50 ml methanol, 75 ml acetic acid, 875 ml water) for 30 min and then electrophoresing them for 1 hour in a Shandon Transverse Disc Destainer (60 ma per gel) with destaining solution in each reservoir.

The length of the gels after destaining and the positions of the blue-stained protein bands were measured.

Mobilities were calculated from the formula:

$$\text{Mobility} = \frac{\text{Distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before destaining}}{\text{distance of dye migration}}$$

A semilogarithmic plot of molecular weight against mobility was used to determine the molecular weights of the membrane proteins (fig 6). Standards (0,01 mg protein) of bovine serum albumin (Sigma, St. Louis, USA), alcohol dehydrogenase (BDH, Poole, England), lactate dehydrogenase (Miles-Seravac, Epping Industria, Cape Town) and cytochrome c (BDH, Poole, England) were electrophoresed as described above.

Absorption profiles of the stained gels were determined at 600 nm with a Gilford (Model 2400) spectrophotometer equipped with a gel scanning attachment (model 2410S).

2.2.9 GRAVIMETRIC DETERMINATION OF TOTAL LIPID CONTENT OF LIPID EXTRACTS.

Aliquots of lipid extract were weighed on a Cahn microbalance as described by Rouser *et al.*(1969).

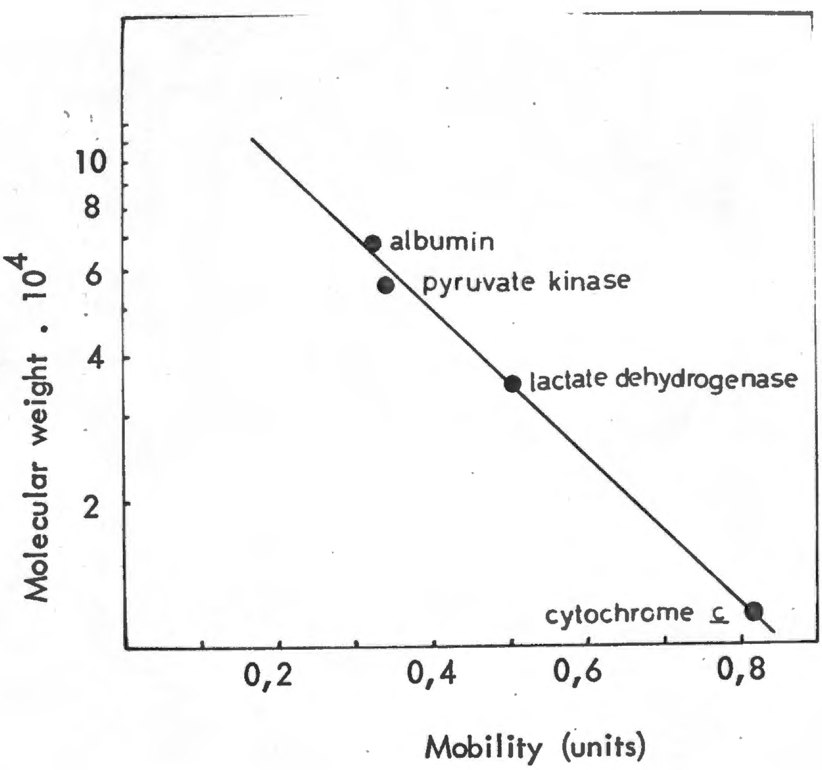


Fig. 6 Standard curve for the determination of molecular weight of proteins.

A mixture of the standard proteins (each approx. 0,01 mg protein) in 0,1% SDS and β-mercoptoethanol together with tracking dye, glycerol, β-mercoptoethanol and dialysis buffer (see 'Methods') was applied to a 10% polyacrylamide gel containing 0,1% SDS. Electrophoresis was performed at a constant current of 8 ma per tube and was continued until the marker dye had moved $^3/4$ of the distance down the tube. The protein bands were stained with Coomassie blue in methanol and 9,2% acetic acid for 5 hrs and destained electrophoretically. Mobility was calculated as

$$\frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before destaining}}{\text{distance of dye migration}}$$

An aluminium pan (5-6 mg) was heated for 3 min at 60-80°C on a melting point block (Gallenkamp, London EC2) cooled over KOH pellets for 2 min in a dessicator and transferred to the microbalance which was adjusted to zero. An aliquot (100-200 μl) of lipid solution was placed in the pan, heated and cooled as before and weighed to $\pm$ 0,1 mg. Heating and cooling periods were carefully timed with a stopwatch and reproduced to within 10 sec.

2.2.10 ELECTRON MICROSCOPY OF FRAGMENTED SARCOPLASMIC RETICULUM.

The negative staining method of Greaser et al.(1969d), a modification of those described by Brenner and Horne (1959) and Huxley (1963) was employed with an AEI EM6B model electron microscope.

A drop of membrane suspension (0,8-1,0 mg/ml), was placed on a parlodion and carbon coated grid held with forceps. The membrane fragments were allowed to settle for 15 sec, and the excess liquid was removed with a small piece of filter paper. A drop of 1% potassium phosphotungstate, pH 7,0 was added immediately and left on the grid for 30-60 sec. The excess stain was removed with filter paper and the grid allowed to dry at room temperature. Staining was carried out at room temperature.

2.3 ENZYME ASSAYS.

2.3.1 CYTOCHROME OXIDASE.

Cytochrome oxidase was used as a marker enzyme in order to detect the presence of fragments or whole mitochondria in purified FSR preparations. Activity was determined by the method of Smith (1955).

Cytochrome c (90 μM in 0,01 M sodium phosphate buffer, pH 7,0) was reduced in the presence of 5% palladium asbestos, by gassing initially with nitrogen for 5 min, hydrogen for 1 hour and again with nitrogen for 5 min. The mixture was rapidly filtered through a Millipore apparatus and used immediately.

Reduced cytochrome c (100 μl of 90 μM) was incubated in a cuvette at 30°C in 0,1 M sodium phosphate buffer (0,5 ml) at pH 7,0. The reaction was initiated by addition of a suspension of FSR (0,05 ml, containing approx. 200 μg protein) and the absorbance change at 550 nm was monitored. The initial rates of change in absorbance per mg protein were compared to those produced by the crude mitochondrial fraction (0,05 ml, containing approx. 20 μg protein) and the results expressed as an apparent percentage contamination of FSR by mitochondria or mitochondrial subparticles. The crude mitochondrial fraction was that pelleted at 8 000 xg for 20 min during the isolation of FSR (see page 58).

2.3.2 SUCCINATE OXIDASE.

Succinate oxidase activity was assayed polarographically (Ernster and Nordenbrand, 1967).

The reaction mixture contained 30 mM sucrose, 50 mM KCl, 25 mM Tris-HCl buffer, pH 7,5, cytochrome c (0,6 mg) and 0,05-0,1 mg mitochondrial protein or 0,1-0,5 mg FSR protein in a final volume of 3 ml. Oxygen uptake was monitored at 30°C by means of a Clark-type electrode (Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio). The reaction was initiated by addition of succinate (15 μl of 1,0 M solution). The initial rates of O_2 uptake per mg FSR protein were expressed as a percentage of the rates obtained in a crude mitochondrial suspension (Section 2.3.1 above).

2.3.3 ATPASE ACTIVITY.

(i) Colorimetric Determination.

The hydrolysis of ATP was carried out in the medium of MacLennan, (1970), which consists of 50 mM Tris-HCl, pH 7,5, 100 mM KCl, 5 mM $MgCl_2$, 0,1 mM EGTA and 0,1 mM $CaCl_2$ (total volume 1 ml). The medium was incubated at 37°C with 50-100 µg FSR protein and the reaction was initiated by the addition of 10 µmoles ATP. The reaction was terminated after 4 min with "silicotungstic acid" solution (4 ml).

The preparation of "silicotungstic acid" solution and the determination of inorganic phosphate released in the reaction was carried out by the Martin and Doty method described by Lindberg and Ernster, (1956).

The silicotungstate solution was prepared by dissolving 16,7 g $Na_2SiO_3.8H_2O$ and 397,0 g $Na_2WO_4.2H_2O$ in 2,5 l distilled water and after adding 75 ml concentrated H_2SO_4 was refluxed for 5 hours. On cooling the mixture was filtered and diluted with distilled water to 5 l.

To determine the concentration of inorganic orthophosphate, 3 ml of sample was acidified with 5 M H_2SO_4 (0,5 ml). An equal mixture of isobutanol and benzene (5 ml) and 10% ammonium molybdate (0,5 ml) was added and the contents well shaken. Separation of the two layers was aided by centrifugation at 1 000 xg for 1 min. Two ml of the top layer was removed with a pipette and diluted with 3,2% (w/v) H_2SO_4 in ethanol (3 ml). Stannous chloride solution (0,5 ml), made by freshly diluting one vol 10% $SnCl_2.H_2O$ in concentrated HCl 50 times with 0,5 M H_2SO_4, was added and mixed immediately. Standards containing 0,05, 0,5 and 1,0 µmoles phosphate were co-determined with the samples. The absorbance was read at 730 nm and a plot of absorbance versus phosphate concentrations was linear.

(ii) Titrimetric Method.

A method in which the release of inorganic phosphate was followed by titration with KOH was developed which is similar to that described by Martonosi and Ferretos (1964b) except that a continuous record of KOH titrant added was included.

The assay medium (total volume 3 ml) consisted of 0,1 M KCl, 5 mM $MgCl_2$, 0,1 mM EGTA, 0,1 mM $CaCl_2$ and 10 mM ATP. The addition of enzyme (50-100 µg FSR protein) initiated the reaction. All solutions were made up freshly with distilled H_2O. The pH was started at pH 7,2 with a TTT1c titrator and a ABU1a autoburette (Radiometer, Denmark) with a 0,2 ml syringe connected to a reservoir of 0,008-0,015 M KOH (CO_2 free). The reaction vessel was continually flushed with CO_2-free nitrogen and thermostated with a water jacket at 37°C. The drive shaft of the titrator was mechanically coupled to a 5 kohm 10 turn retransmitting potentiometer which was connected to a 10 inch strip chart recorder of 50 mV sensitivity. Chart speed was 0,5 inch per min.

The rate of inorganic phosphate released was calculated on the basis that 1 µmole ATP hydrolysed yields 0,7 µmoles H^+ (Martonosi and Ferretos, 1964b). The recorder response was set such that full scale deflection was equivalent to 0,2 µmoles inorganic phosphate released. Non-enzymatic hydrolysis of ATP was found to be insignificant in the temperature range 0-50°C. In some experiments the temperature of the circulating water bath was allowed to rise from 10°C to 55°C and the ATPase activity was continuously monitored. The temperature of the reaction mixture during the heating was monitored with a thermistor probe (Yellow Springs Instrument Co, Inc., Yellow Springs, Ohio). Corrections were made for temperature dependence of the pH electrode.

2.3.4 PHOSPHORYLATION OF ATPASE.

The method is based on that of Martonosi (1969) and MacLennan(1970).

The assay system contained 100 µmoles KCl, 10 µmoles histidine, pH 7,5, 10 µmoles each of $CaCl_2$ and $MgCl_2$, and approximately 1 mg protein in a total volume of 2 ml.

The reaction was initiated by the addition of 0,05 µmoles ATP labelled in the γ- position with ^{32}P (0,5mCi/ml; Radiochemical Centre, Amersham, England) and stopped after 20 sec by the addition of ice-cold 5% trichloracetic acid (35 ml) containing 0,1 mM ATP and 1 mM phosphate. Following centrifugation at 30 000 xg for 1 hour the supernatant was decanted and the sediment was washed once with 5% trichloracetic acid (35 ml) and twice with 2% trichloracetic acid (10 ml). The final sediment was suspended in a solution of 0,1 M NaOH and 2% Na_2CO_3 (1 ml) and heated for 15 min in a boiling water bath. Aliquots (0,2 ml) were taken for analysis of radioactivity and protein.

2.4 CALCIUM BINDIND AND TRANSPORT.

2.4.1 ATP-DEPENDENT CALCIUM BINDING BY FRAGMENTED SARCOPLASMIC RETICULUM.

The method is essentially that described by Martonosi and Ferretos (1964a) using a 'Millipore' filter system and ^{45}Ca.

The assay medium (1 ml) consisted of 20 mM histidine, pH 7,4, 50 mM KCl, 5 mM $MgCl_2$, 0,5 mM EGTA, 0,5 mM $^{45}CaCl_2$ (0,2 m Ci/ml; Radiochemical Centre, Amersham, England) and 5 mM ATP. The reaction, conducted at 25°C, was initiated by addition of 100-300 μg protein and terminated after 2 min by filtering an aliquot (0,2 ml) through a 0,45 μ Millipore filter (type HA) and washing with 0,1M KCl, 5 mM histidine, pH 7,4 (5 ml). The reaction was considered terminated as soon as washing commenced. When the time-course of binding was followed the reaction was accurately timed with a stop watch and the first aliquot was filtered and washed within 7 sec of initiation. Further aliquots were filtered and washed at approximately 12 sec and at 60 sec and 180 sec. The amount of calcium bound per mg protein was calculated from the amount of radioactivity remaining on the filter. The filter disc was placed in liquid scintillation fluid (5 ml) consisting of 0,8% ($^{w}/v$) Fluoralloy (formula TLC of Beckman Instruments, Inc., Palo Alto, California) and 2,5% ($^{w}/v$) Bio-solve (formula BBS-3, Beckman Instruments) in toluene and counted in a Beckman Liquid Scintillation Counter, model LS-233.

Controls containing no ATP were also assayed with each determination and subtracted from the test values. Thus the results show only ATP dependent binding (it is also understood that the non-exchangeable Ca^{2+} normally present in FSR, see results page 150, does not contribute to the measurable amount of Ca^{2+} bound by this method).

2.4.2 ATP-DEPENDENT CALCIUM UPTAKE BY FRAGMENTED SARCOPLASMIC RETICULUM.

The assay is similar to that described under ATP-Dependent Calcium Binding except that 5 mM K-oxalate was included in the assay medium and the timing of aliquots as indicated in the individual experiments (usually 0,5 1, 5, 10 and 15 min).

2.4.3 PASSIVE CALCIUM BINDING BY FRAGMENTED SARCOPLASMIC RETICULUM.

The method was adapted from that described by Carvalho (1966).

All operations were conducted at room temperature (approximately 20°C). Suspension of FSR (0,1 ml containing approx. 0,5 mg protein) in 0,1M KCl, 5 mM histidine, pH 7,2, was added to 10,0 ml of double-distilled deionised water and the pH adjusted to 7,00 with 0,01M KOH by means of a TTT1c titrator (Radiometer, Copenhagen, Denmark) with continual magnetic stirring. The final concentration of K^+ ions was less than 1 mM which included a small contribution from the stock FSR suspension. Calcium was added with a 10 µl Hamilton syringe from a stock solution of either 0,01M or 0,1M $CaCl_2$ to give range of nominal concentrations from 0-100 µM. The suspension was adjusted to pH 7,00 and equilibrated for at least 15 min before centrifugation at 105 000 xg for 10 min. The supernatant was decanted and the calcium concentration determined. The walls of the centrifuge tube and the surface of the pellet were rinsed with water twice and the solution discarded. The pellet was dispersed in water (1 ml) in the centrifuge tube by sonicating with an Ultrasonics Rapidis 300 (Ultrasonics Ltd., Shipley, Yorks, England) sonicator equipped with a 0,9 cm diameter probe at the first noise level for 10 sec. The protein (Lowry method) and calcium concentrations were determined.

The calcium content was measured by a Varian-Techtron Atomic Adsorption Spectrophotometer type AA-5 using a nitrous oxide and acetylene flame. The concentration of calcium was graphically determined from the standard curve in fig. 7 in which recorder response is plotted against calcium concentration (10-90 µM).

2.4.4 PASSIVE CALCIUM EFFLUX FROM FRAGMENTED SARCOPLASMIC RETICULUM.

(i) Energized Preloading.

Vesicles (approx. 1,0 mg protein) were maximally loaded at 25°C with ^{45}Ca, using the Ca-Uptake medium (2 ml), for 20 min. EDTA (final conc 6 mM)was added to inhibit the calcium transport enzyme and the passive efflux was monitored by filtering aliquots (0,2 ml) every 5 min on the millipore apparatus.

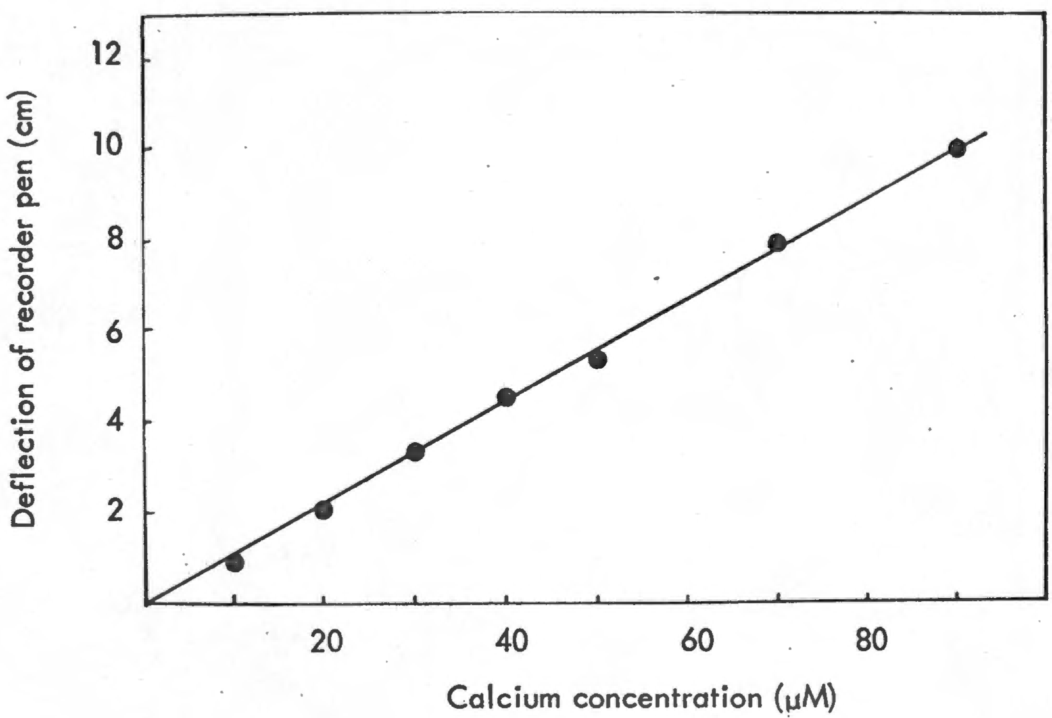


Fig. 7 Standard curve for determination of calcium by atomic absorption spectrophotometry.

Standard solutions of Ca^{2+} were prepared in 1% $LaCl_2$. Absorption of emission from the Ca^{2+} lamp was determined at 422.7 nm in a nitrous oxide-acetylene flame.

The radioactivity on the filter disc was counted as described under Ca-Binding.

(ii) Passive Preloading.

The method was adapted from that described by Inesi *et al.* (1973).

FSR (6-8 mg protein/ml) was preincubated overnight with 0,1 M KCl, 5 mM histidine, pH 7,0, 5 mM $MgCl_2$ and 20 mM $^{45}CaCl_2$ (0,2 mCi/ml; Radiochemical Centre, Amersham, England). Preincubation was carried out at 2-4°C. Efflux was initiated by a 20 fold dilution of the 'loaded' FSR vesicles with diluting medium previously equilibrated at 25°C. The diluting medium, 0,1 M KCl, 5 mM histidine, pH 7,0, 5 mM $MgCl_2$ and 10 mM EGTA, initiated passive efflux. Efflux was followed by filtering aliquots (0,2 ml) at various times and determining residual radioactivity on the filter as described under ATP-Dependent Calcium Binding.

.2.4.5 COUPLED EFFLUX OF CALCIUM FROM FRAGMENTED SARCOPLASMIC RETICULUM.

The method is as described under section 6.4.4 (ii) except that the diluting medium included 1,0 mM ADP and 3,0 mM Pi (Inesi *et al.*, 1973).

3.0 RESULTS.

3.1 CRITERIA FOR RECOGNITION AND CHARACTERISATION OF SLOWLY- AND OF RAPIDLY-GLYCOLYSING MUSCLE.

Summary: Muscle selected as slowly-glycolysing (normal) was red and firm and it exuded little fluid. Muscle judged to be rapidly-glycolysing (abnormal) was obviously pale and soft and it exuded considerable amounts of fluid. The pH of slowly-glycolysing and pale, soft, exudative (PSE) muscle at the time of excision was approximately 6,7 and 5,7 respectively. The glycogen and lactate contents of the two muscle types differed significantly (25,8 versus 1,70 μmoles glucose equivalents/g wet muscle and 48,6 versus 107,5 μmoles lactate/g wet muscle for normal and abnormal muscle respectively).

Skeletal muscle (longissimus dorsi) was characterised as either normal or having undergone pale, soft, exudative change (PSE) on the basis of colour, texture, consistency and pH (table 1). Only muscle that satisfied all four criteria unequivically for either normal or PSE types was selected for further investigations. The average pH of muscle selected as PSE was approximately 1,0 units lower than normal, slowly-glycolysing muscle. Confirmation of the muscle type was achieved by analysis of skeletal muscle glycogen and lactate contents. The glycogen content of PSE muscle was diminished to near zero values whilst the lactate concentration was.. more than double. Fig 8 depicts samples of slowly-glycolysing and PSE muscle in which the differences in colour and exudation are obvious.

slowly-glycolysing (normal) muscle

rapidly-glycolysing (abnormal or PSE) muscle

Fig. 8 Samples of slowly-glycolysing muscle and of PSE muscle.

Tissue was excised from Longissimus dorsi muscle of exsanguinated pigs at 10–15 min *post-mortem*. The muscle was immediately placed in ice and transported to the laboratory ($\pm$ 35 min) and photographed within 15 min of arrival. At the time of photographing, the samples of slowly-glycolysing muscle and of PSE muscle had pH values of 6,63 and 5,65 respectively.

exudation from slowly-glycolysing muscle.

exudation from PSE muscle.

Fig. 9 The exudation from slowly-glycolysing and PSE muscle.

The exudation is that remaining on the filter paper after the muscles samples shown in fig. 8 were removed.

Table 1 Parameters used to assess muscle type

Assessment of colour, texture and consistency of muscle was subjective. The overall pH of a muscle was a mean of three readings taken at sites in the muscle approximately 4 cm apart. For glycogen and lactate determinations, muscle samples (0,5–1,5 g) were placed in 10% ($^w/_v$) trichloracetic acid and 50% ($^w/_v$) perchloric acid, respectively, immediately on excision from the carcass (see Methods for the procedure for glycogen and lactate determinations). Muscle samples were excised 10–15 min *post-mortem*. The results are expressed as mean $\pm$ S.D. (n).

Muscle type	Colour	Texture	Consistency	pH	Glycogen (µmoles glucose equivalents, g wet tissue^{-1})	Lactate (µmoles lactate g wet tissue^{-1})
Normal	red	firm	moist	6,73 $\pm$ 0,14 (10)	25,8 $\pm$ 9,9 (7)	48,6 $\pm$ 15,5 (10)
PSE	pale	soft	watery	5,69 $\pm$ 0,36 (10)	1,70 $\pm$ 1,22 (8)	107,5 $\pm$ 18,5 (10)

3.2 ISOLATION OF FRAGMENTED SARCOPLASMIC RETICULUM.

Summary: Centrifugation of a crude microsomal fraction prepared from skeletal muscle, through a discontinuous sucrose gradient of 45, 40 and 35% ($^w/v$) sucrose resulted in two bands located at the two interfaces and another on the surface of the gradient. The crude material from PSE muscle only banded at the 40-35% sucrose interface and at the surface of the gradient. The lower band with high bouyant density was consistently absent. Approximately 52 mg crude microsomal material/100g wet muscle was routinely recovered from both muscle types. Electron photomicrographs of the material in each band showed it to be composed largely of vesicles characteristic of FSR.

Crude microsomal material (see Methods) freed of contaminating actomyosin by dissolution with 0,6M KCl, was pelleted by centrifuging at 30 000 xg for one hour and suspended in 0,1M KCl, 5 mM histidine, pH 7,2. It was then layered on a discontinuous sucrose gradient of 35, 40 and 45% ($^w/v$) and centrifuged for 2 hours at 25 000 rpm. Figure 10 shows the banding pattern obtained with material derived from slowly-glycolysing muscle and from PSE muscle. The preparations from slowly-glycolysing muscle banded in three distinct regions, namely at the 40-45% sucrose interface, the 35-40% sucrose interface and at the top of the 35% sucrose layer. These bands have been labelled ; N_1, N_2 and N_3 respectively.

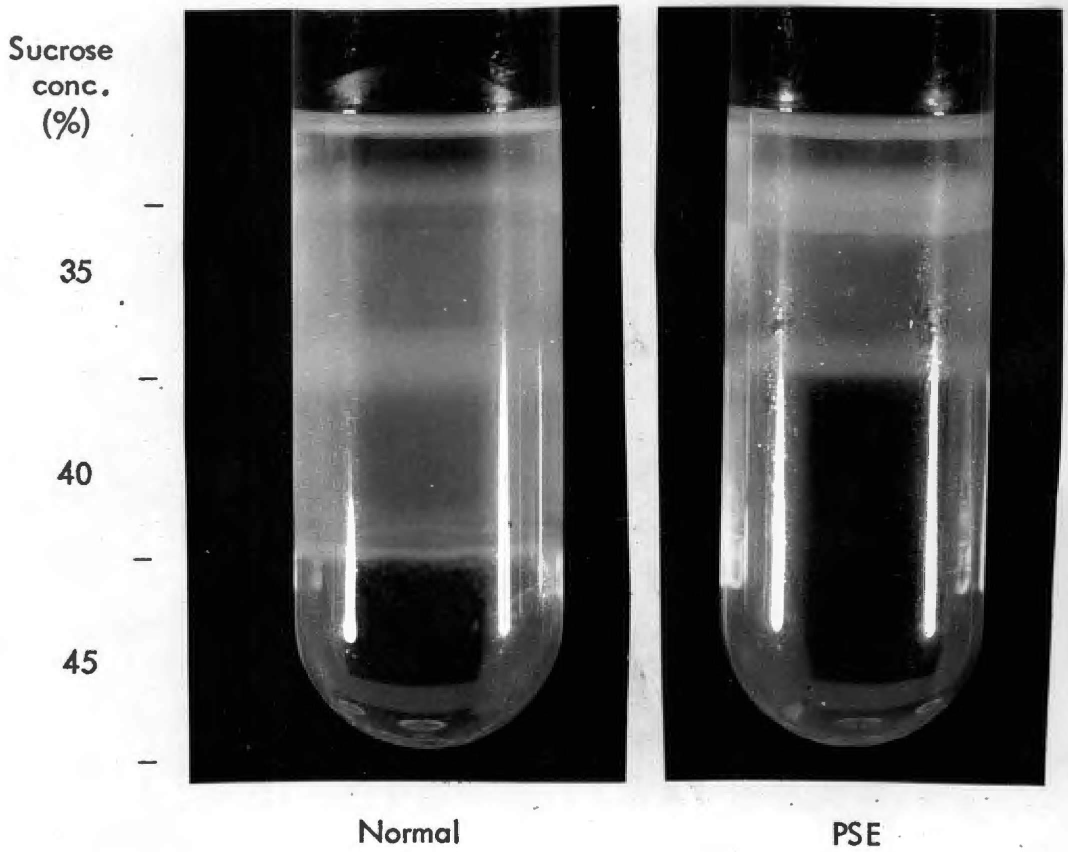


Fig. 10 Sedimentation behavior in discontinuous sucrose gradients of a crude microsomal fraction isolated from slowly-glycolysing muscle and from PSE muscle.

Homogenates (50g muscle in 200 ml 0,1M KCl, 5 mM histidine, pH 7,2) of slowly-glycolysing muscle and of PSE muscle were subjected to differential centrifugation and the microsomal fraction isolated by centrifugation at 30 000 xg for 1 hour. The pellets were suspended in 0,1M KCl, 5 mM histidine, pH 7,2 (approx. 25 mg protein/ml) and the suspensions (2 ml) were layered onto discontinuous sucrose gradients of 35%, 40% and 45% ($^{w}/v$) sucrose. Centrifugation at 64 700 xg for 2 hours resulted in the sedimentation pattern shown.

The pattern is very similar to that obtained by Greaser et al. (1969 d) for microsomal preparations from pig and rabbit skeletal muscle, using the same isolation and purification procedure. PSE preparations banded in only two regions, namely at the 35-40% sucrose interface and at the top of the 35% sucrose layer. The two bands were labelled P_2 and P_3 respectively. Particulate material was consistently absent from the 40-45% interface. In some preparations, the 35-40% sucrose interface contained only a small quantity of visible material and most of the crude FSR preparation remained at the top of the 35% sucrose layer.

The lower band of the normal preparation was aggregated and dense. The middle and top bands were diffuse and not aggregated. P_2 varied in appearance. A thin dense band often demarkated its lower edge. The proportion of protein recovered from each band varied considerably from one preparation to another. The average recovery of 14 isolation procedures are given in table 2. There was no difference in the amount of protein recovered from gradients prepared from slowly-glycolysing and from PSE muscle. An average of 52 mg FSR protein was recovered from 100g wet weight of muscle. Approximately one-half of this protein sedimented at the 35-40% sucrose interface with the remainder equally distributed between the extreme bands. The proportion of protein varied between the two bands of the PSE gradient. An average of 62% of the protein applied to the gradient was recovered above the 35% sucrose layer ($\rho <1$, 158 g/ml).

Possible contamination of each band by mitochondria was investigated by assaying the activities of both cytochrome oxidase and succinate oxidase (table 3).

Table 2 Recovery of protein from discontinuous sucrose gradients of crude FSR from slowly-glycolysing (normal) and from PSE muscle.

The homogenised muscle was subjected to differential centrifugation and the crude microsomal fraction obtained. The microsomal material (approx. 50 mg protein in 2 ml 0,1M KCl, 5 mM histidine, pH 7,2) was centrifuged through a discontinuous sucrose gradient of 35, 40 and 45% ($^w/v$) sucrose at 64 700 xg for 2 hours. The material banded at 40-45% sucrose interface, 35-40% sucrose interface and on the surface of the 35% sucrose layer. The bands were designated N_1, N_2 and N_3 respectively if the muscle had been normal and P_1, P_2 and P_3 respectively if the muscle had been PSE. The material was eluted through a puncture hole at the bottom of the centrifuge tube by the application of air pressure at the top. Protein was determined by the Biuret method. The results are expressed as mean $\pm$ S.D. (n=14).

Fraction	Protein Recovery (mg/100g wet muscle wt.)	
	Normal	PSE
3	15,72 $\pm$ 10,26	31,58 $\pm$ 15,26
2	26,22 $\pm$ 16,80	19,08 $\pm$ 16,44
1	14,30 $\pm$ 8,76	$<$0,2

Table 3 Mitochondrial contamination of purified fractions of fragmented sarcoplasmic reticulum isolated by discontinuous sucrose density centrifugation.

Cyt.oxidase activity of membrane suspensions (approx. 0,1 mg protein) was measured at 550 nm in the presence of 50 µmoles sodium phosphate buffer, pH 7,0 and 9 nmoles reduced cyt c (in a total vol. of 0,65 ml). A polarographic method was used to determine succinate oxidase activity. The O_2 consumed by membrane suspensions (0,1-0,3 mg of protein) was measured in the presence of 90 µmoles sucrose, 150 µmoles KCl and 75 µmoles Tris-HCl buffer, pH 7,5 (total vol. 3 ml). In both assays the initial rate of reaction was expressed as a percentage of that of a mitochondrial suspension. The mitochondrial fraction represents crude material pelleted after 20 min centrifugation at 8 000 xg after an initial centrifugation of 1 000 xg for 20 min to remove the heavier particles.

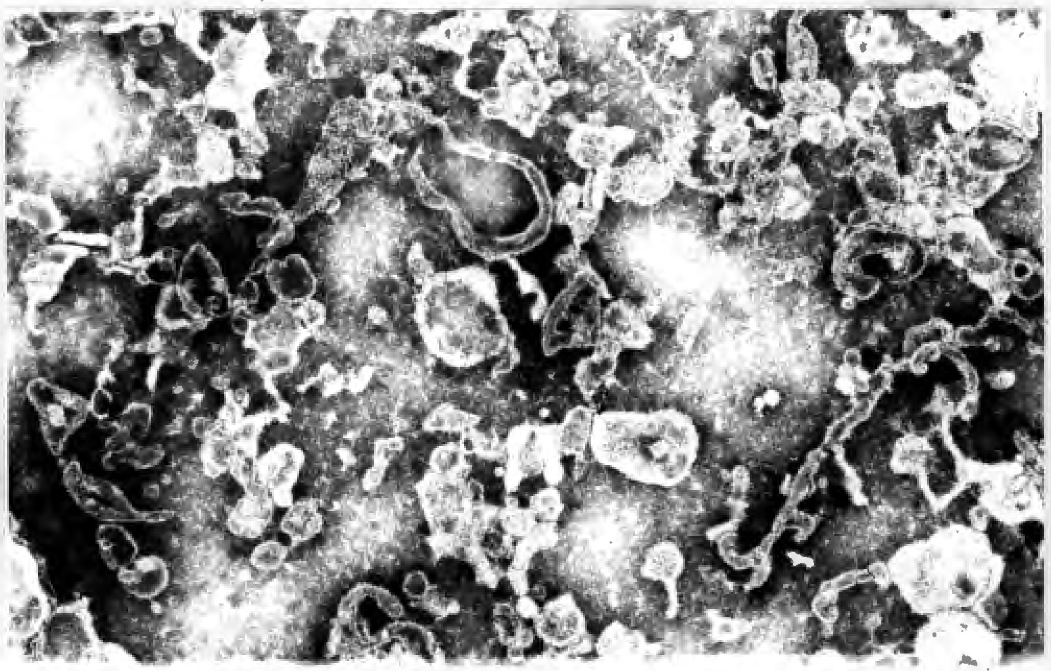
	Percentage Contamination	
Fraction	Cytochrome Oxidase Activity	Succinate Oxidase Activity
N_3	0,3	0,4
N_2	5,6	0,9
N_1	6,0	1,1
P_3	1,9	1,4
P_2	7,6	3,1
P_1	-	-

Succinate oxidase activity was minimal in all bands (corresponding to approx. 1%g weight of contaminating mitochondria). Cytochrome oxidase activity rose towards the denser regions of the gradient so that N_1 displayed 6,0% and P_2 7,6% of mitochondrial contamination. The PSE fractions were more contaminated by mitochondria than were normal fractions. It should be noted that the mitochondrial suspension (see legend to table 3 for details) was not purified.

Electron micrographs (fig. 11) of the material banding in the gradients (fig. 10) revealed that all fractions were composed predominantly of vesicular material which was consistent in size and shape with the descriptions of FSR vesicles derived from pig and rabbit skeletal muscle (Greaser et al., (1969 d). Some mitochondrial fragments with typical spheres 90 Å diameter lining the membrane surface are apparent in N_3. The diameter of the vesicles varied from 600 to 1300 Å and the thickness of the delimiting membrane varied from 90 to 120 Å.

Two different types of vesicles could be identified on the basis of the electron density of their centres. Vesicles with electron-transparent centres have well-defined lining membranes. Another clearly distinguished type of vesicle has an electron-opaque centre, less well-defined lining membrane and many such vesicles have elongated processes or 'tails'. These so-called 'tadpole tails' have recently been identified as artefacts of the isolation procedure (Baskin, 1971). The proportion of the two types differed in the different fractions. The material of heaviest bouyant density (N_1) from slowly-glycolysing muscle consisted predominantly of electron-transparent vesicles, whilst the material of intermediate bouyant density contained predominantly smaller electron-opaque vesicles.

(a)



(b)

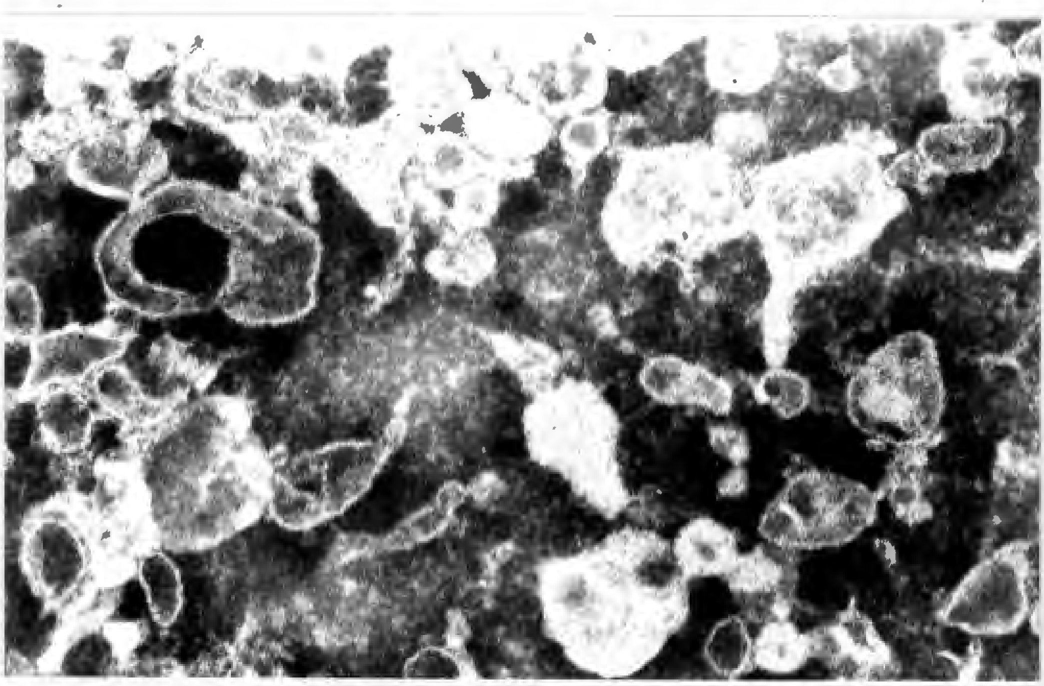


Fig.11 Electron photomicrographs of fragmented sarcoplasmic reticulum.

The crude microsomal fraction from slowly-glycolysing muscle and from PSE muscle separated on a discontinuous sucrose gradient (see fig. 10), into three (N_1, N_2 and N_3) and two (P_2 and P_3) fractions respectively. The crude microsomal fraction and each of the purified fractions were examined with an electron microscope. (a) crude microsomal fraction from slowly-glycolysing muscle, 60 000 x and (b) 120 000 x .

(c)

(d)

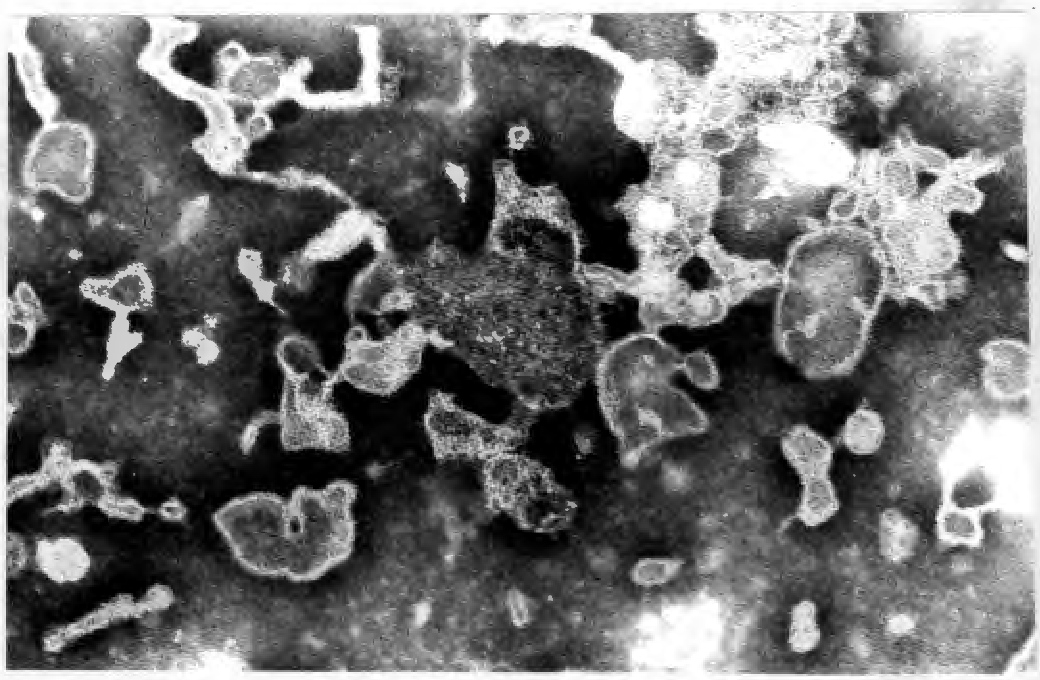
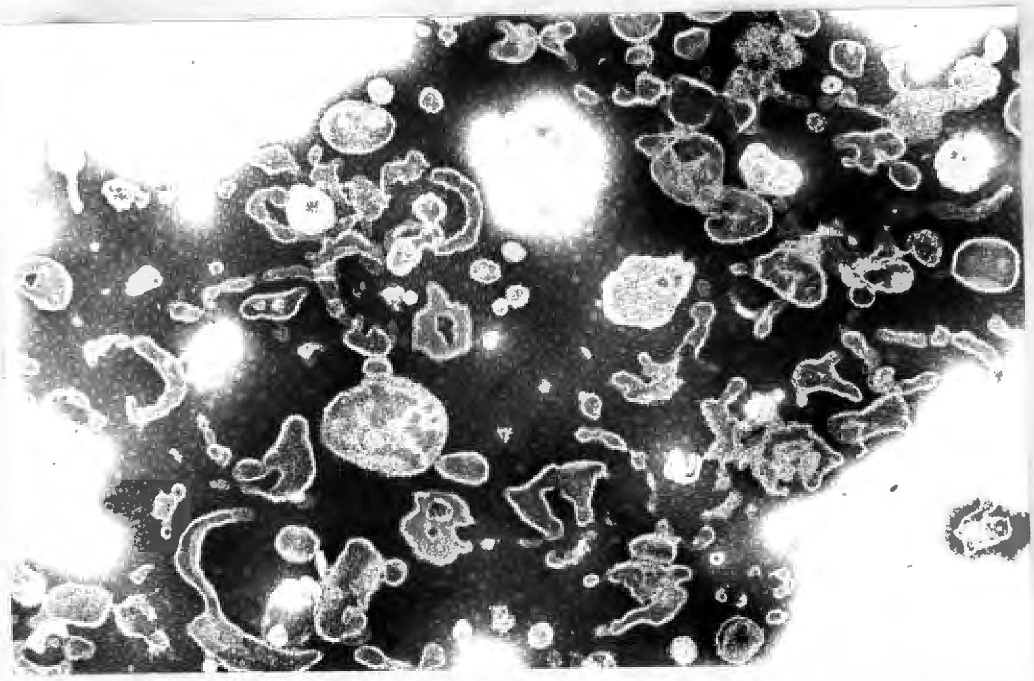


Fig. II cont. (c) crude microsomal fraction from PSE muscle, 60 000 x and (d) 120 000 x .

(e)



(f)

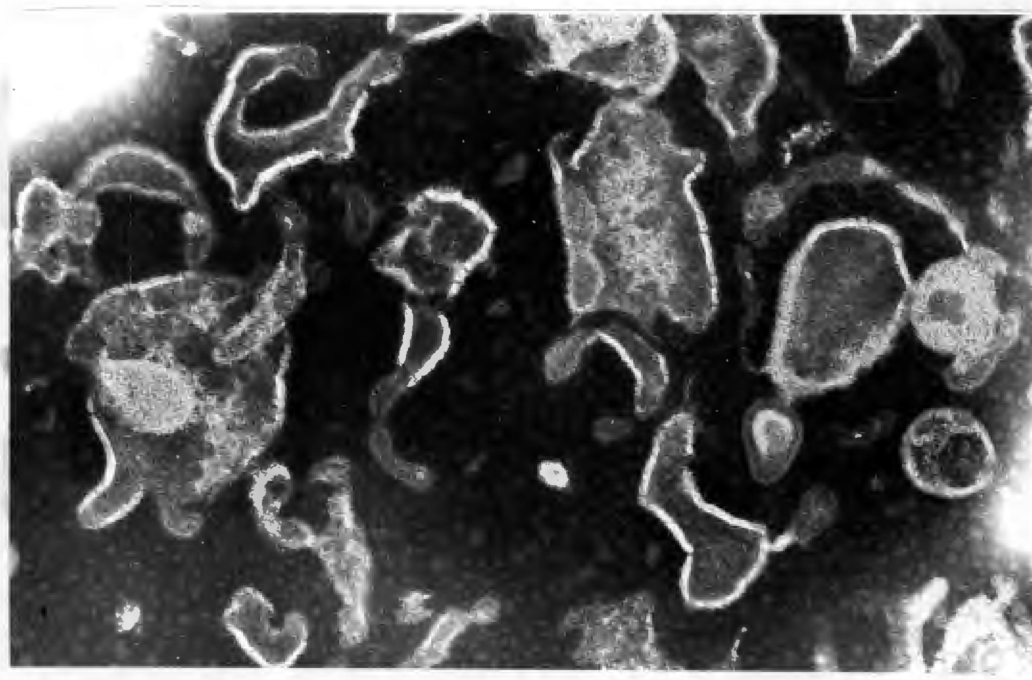
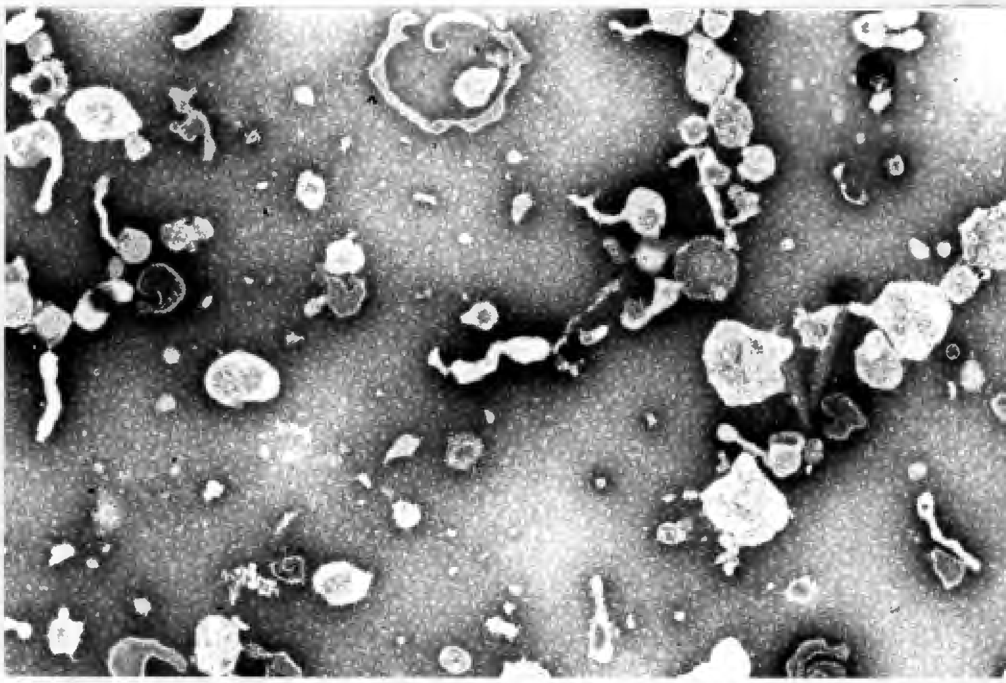


Fig. 11 cont. (e) N_1 fraction of FSR from slowly-glycolysing muscle, 60 000 x and (f) 120 000 x .

(g)



(h)

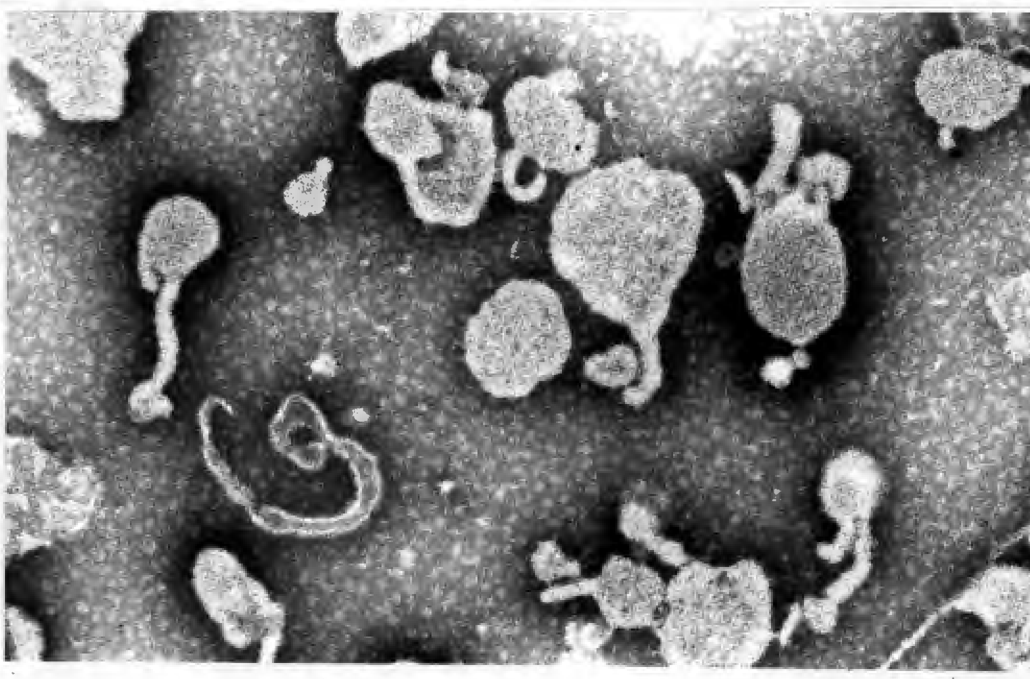
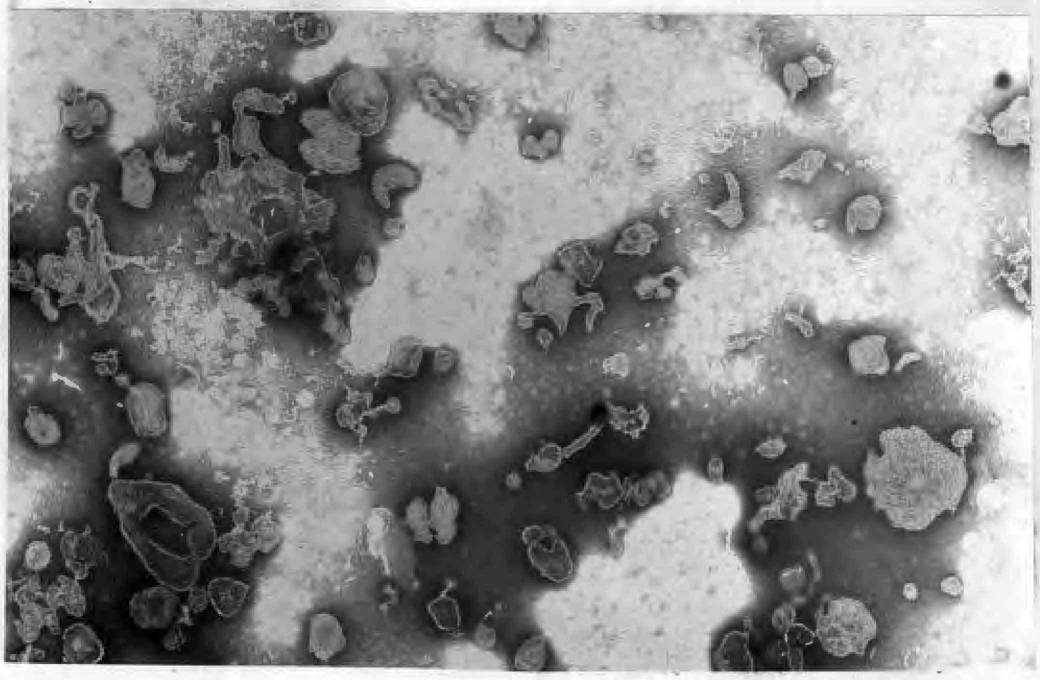


Fig. 11 cont. (g) N_2 fraction of FSR from slowly-glycolysing muscle, 60 000 x and (h) 120 000 x .

(i)



(j)

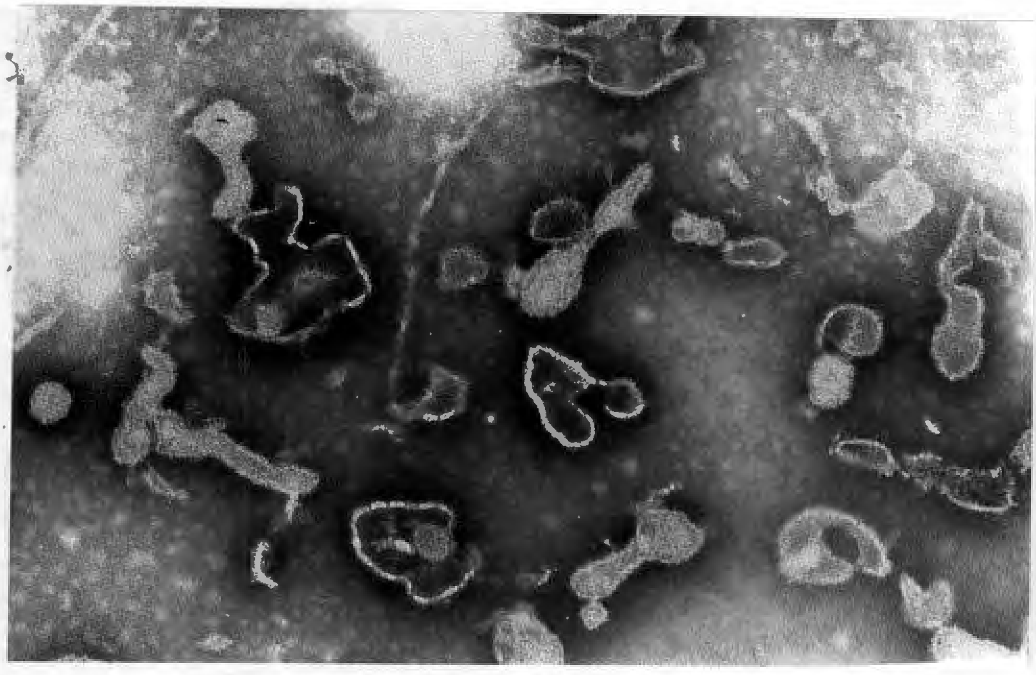
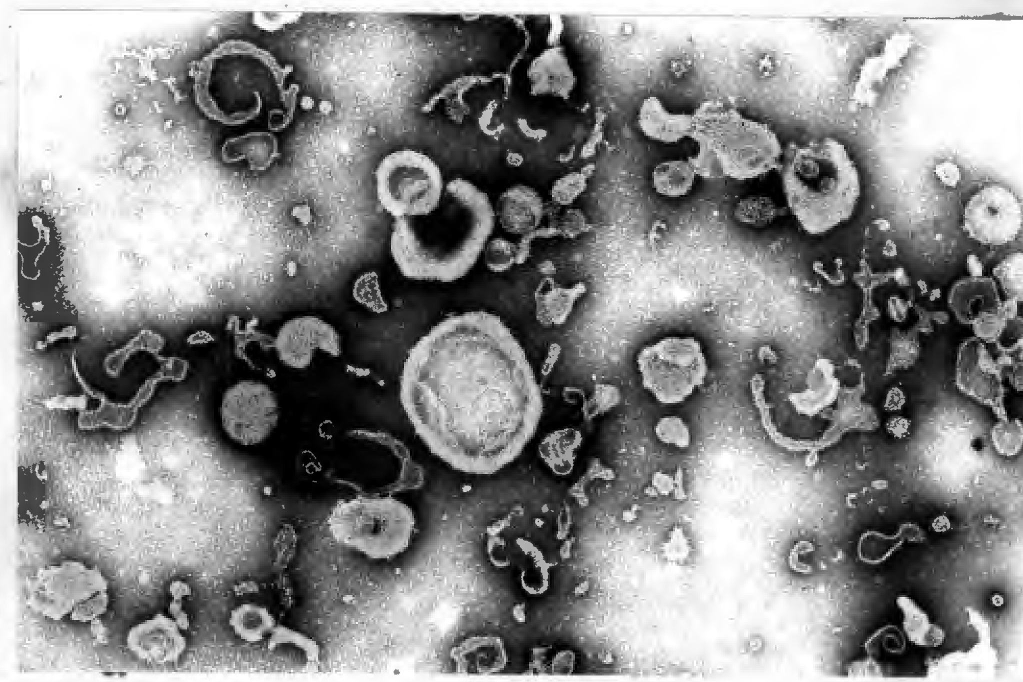


Fig. 11 cont. (i) P_2 fraction of FSR from PSE muscle, 60 000 x and
(j) 120 000 x .

(k)



(l)

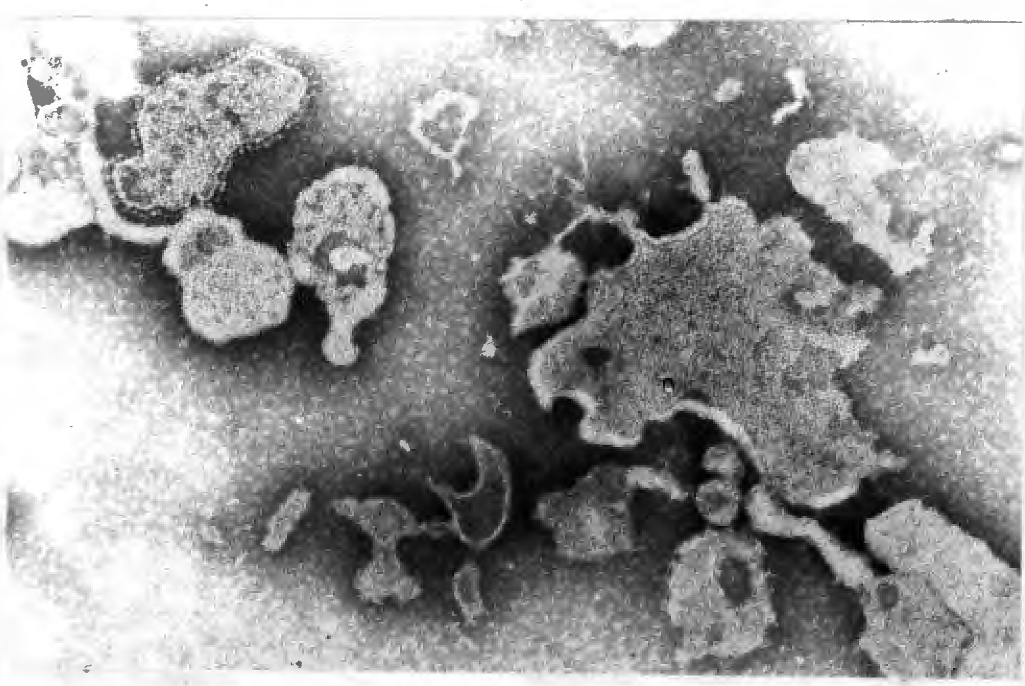
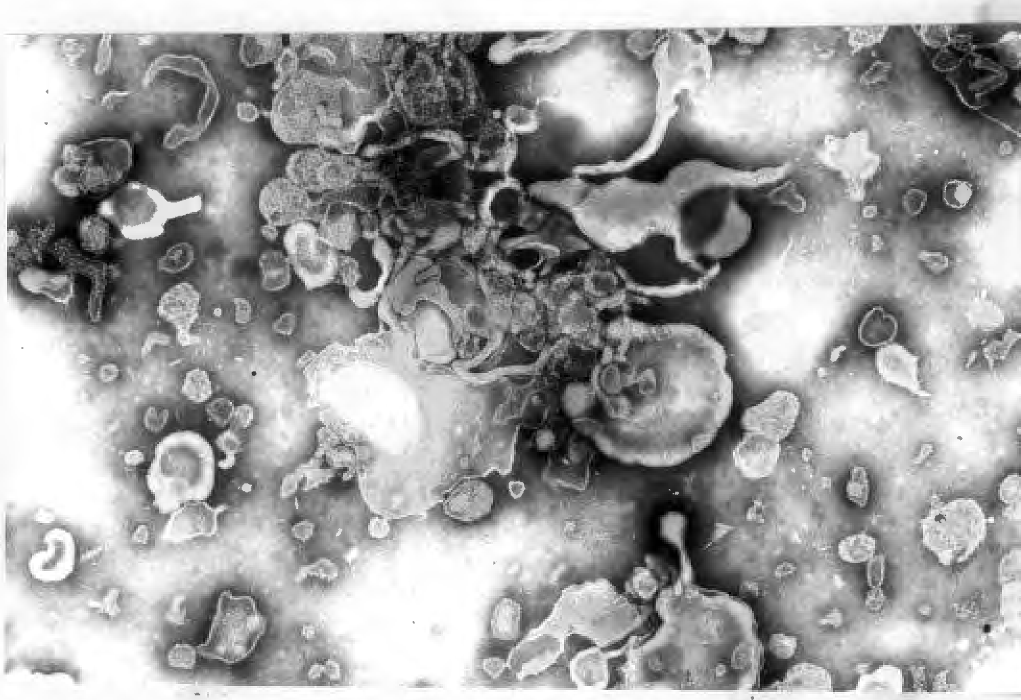


Fig. 11 cont. (k) N_3 fraction of FSR from slowly-glycolysing muscle, 60 000 x and (l) 120 000 x .

(m)



(n)

Fig. 11 cont. (m) P_3 fraction of FSR from PSE muscle, 60 000 x and (n) 120 000 x .

The material of lowest bouyant density (N_3) comprised approximately equal quantities of each vesicle type. The two fractions of purified FSR isolated from PSE muscle contained approximately equal quantities of both types of vesicles. The vesicular material from all fractions tended to aggregate in clumps. Material of highest bouyant density whether from normal, slowly-glycolysing muscle (N_1) or rapidly glycolysing PSE muscle (P_2), exhibited an increased tendency to aggregation.

Recentrifugation of the purified fractions N_1, N_2 and N_3, in low density buffer (0,1M KCl, 5 mM histidine, pH 7,2) resulted in a pellet which was easily dislodged from the wall of the centrifuge tube on standing (fig 12). The comparable material from PSE muscle (P_2, P_3) remained firmly attached to the wall of the tube under these conditions.

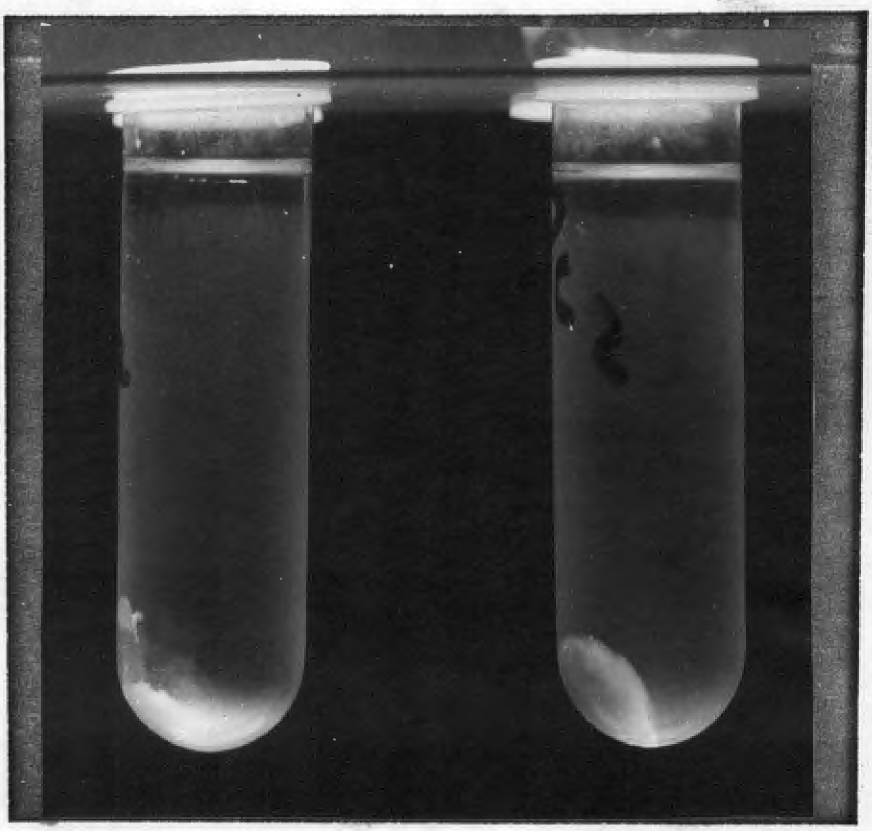


Fig. 12 Aggregation of purified, fragmented sarcoplasmic reticulum from slowly-glycolysing muscle and from PSE muscle.

The material, harvested from N_2 and P_2 fractions was dispersed in 0,1M KCl, 5 mM histidine, pH 7,2, (30 ml) and centrifuged at 78 100 xg for 45 min. The tubes stood for 10 minutes at 2–4°C and were then photographed.

3.3 FUNCTIONAL ACTIVITIES OF THE FRAGMENTED SARCOPLASMIC RETICULUM.

Summary: All purified fractions of membranes isolated from slowly-glycolysing muscle and from PSE muscle on discontinuous sucrose gradients displayed calcium-accummulating ability and ATPase activity. The most active material (N_2) showed a capacity and transport rate for calcium approximately 4 times that of the corresponding fraction from PSE muscle, P_2 (38,3 versus 13,0 nmoles Ca^{2+}. mg protein^{-1} in the absence and 570,9 versus 121,0 nmoles Ca^{2+}/min. mg protein^{-1} in the presence of 5 mM oxalate). The two extreme bands from slowly-glycolysing muscle (N_1 and N_3) also exhibited greater capacities and transport rates for calcium than both the purified fractions from PSE muscle. ATPase activity of N_2 was higher than that of P_2 (1,66 versus 1,13 µmoles Pi/min. mg protein^{-1}) but the difference was not as great as that between the calcium transport parameters. The ATPase activity of the least dense material in N_3 and P_3 was similar. The extent of formation of phosphoprotein intermediate in fragments N_2 and P_2 was similar.

The sarcoplasmic reticulum has one clearly-defined function, namely the regulation of intracellular calcium concentration. Isolated sarcoplasmic reticulum spontaneously forms vesicles that can actively accummulate calcium against a concentration gradient (fig 13). Without an intravesicular agent for precipitating calcium, FSR from porcine muscle is able to bind approximately 38 nmoles calcium per mg protein (table 4). This process is extremely rapid at 25°C and the millipore filtration technique used here is unable to measure initial rates of Ca-Binding (fig 13).

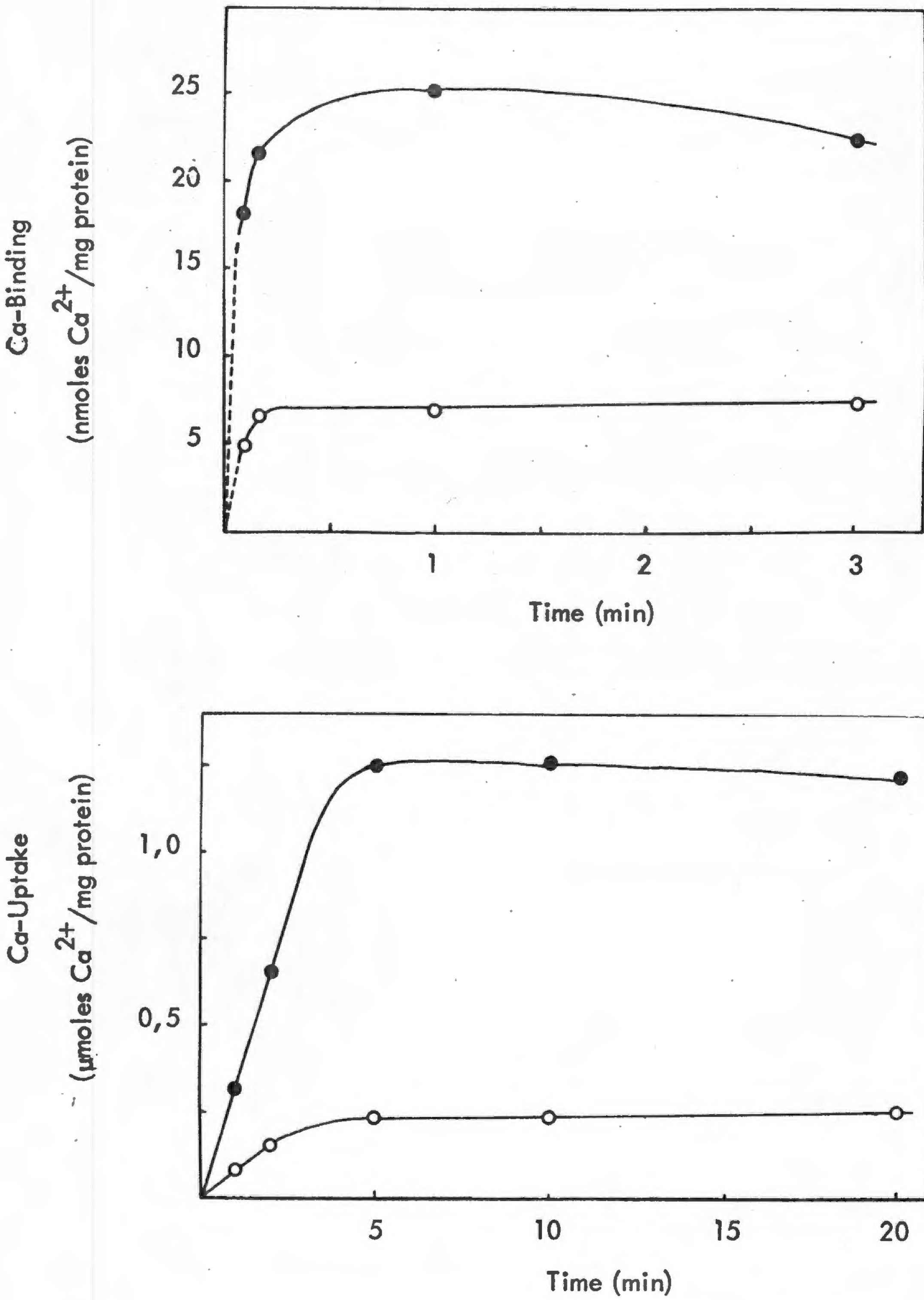


Fig. 13 <u>Time course of Ca-Binding and Ca-Uptake of calcium by fragmented sarcoplasmic reticulum</u>.

Ca-Binding was determined at 25°C with FSR suspension (0,13-0,30 mg protein) from N_2 and P_2 fraction ($1{,}158 < \rho < 1{,}184$ g/ml) in a medium of 20 mM histidine-HCl, pH 7,4, 50 mM KCl, 5 mM $MgCl_2$, 0,5 mM EGTA, 0,5 mM $^{45}CaCl_2$, and 5 mM ATP (total vol. 1 ml). Ca-Uptake was determined with double the amount of FSR suspension in a medium similar to that for Ca-Binding but it contained in addition 5 mM oxalate. The total volume was 2 ml. Aliquots were removed at various time intervals and filtered through a millipore system. The filter was washed with 0,1M KCl, 5 mM histidine buffer, pH 7,4, and the radioactivity remaining on the filter disc counted in a liquid scintillation counter. FSR from slowly-glycolysing muscle (●) and from PSE muscle (o).

Table 4 Phosphoprotein intermediate formation, adenosine triphosphatase activity and calcium accumulating ability of purified fragmented sarcoplasmic reticulum.

The results are means $\pm$ S.D. of 7 preparations except for phosphoprotein intermediate formation which are means of duplicate determinations on a single preparation. Formation of phosphoprotein intermediate was measured at 25°C over 20 sec in a medium containing 50 mM KCl, 0,5 mM histidine, pH 7,5, 0,5 mM $CaCl_2$ and $MgCl_2$, 25 µM ATP (γ^{32}P) and 0,8-1,3 mg protein (total vol. 2 ml). The reaction was terminated by the addition of 5% (w/v) TCA, 0,1 mM ATP and 1 mM phosphate (35 ml). The protein precipitate was pelleted, washed thrice with TCA solutions (5%, 2% and 2%) and finally dissolved in 0,1 M NaOH, 2% Na_2CO_3 (1 ml). Aliquots (0,2 ml) were taken for protein determination and ^{32}P determination by scintillation counting. ATPase activity was measured at 37°C in a medium of 50 mM Tris-Cl, 100 mM KCl, 5 mM $MgCl_2$, 0,1 mM EGTA, 0,1 mM $CaCl_2$, 10 mM ATP and 0,07-0,18 mg FSR protein suspension. ATP hydrolysis was terminated by the addition of "silicotungstic acid"solution (4 ml) (see "Methods"). Inorganic phosphate was measured as reduced phosphomolybdate (see "Methods"). Ca-Binding and Uptake was determined as in fig. 13 except the reaction in each case was terminated after 2 min.

Membrane Fraction	Phosphoprotein Intermediate Formation (moles ATP. 10^6g protein^{-1})	ATPase Activity (µmoles Pi/min. mg protein^{-1})	Calcium Accumulation: Binding (nmoles Ca^{2+} mg protein^{-1})	Calcium Accumulation: Uptake (nmoles Ca^{2+}/min. mg protein^{-1})
N3	0,93	0,90 $\pm$ 0,22	40,9 $\pm$ 7,1	444 $\pm$ 73
P3	0,61	0,97 $\pm$ 0,25	17,7 $\pm$ 8,7	136 $\pm$ 22
N2	1,15	1,66 $\pm$ 0,18	38,3 $\pm$ 12,8	570 $\pm$ 94
P2	0,84	1,13 $\pm$ 0,20	13,0 $\pm$ 4,8	121 $\pm$ 26
N1	1,27	1,39 $\pm$ 0,30	20,0 $\pm$ 15,2	256 $\pm$ 10
P1	-	-	-	-

However, in the presence of oxalate which prevents the accumulation of limiting levels of calcium ions by the intravesicular precipitation of Ca-oxalate, linear transport continues for at least 2 min at 25°C, permitting measurement of initial transport rates (fig. 13). In addition, at least 10 times more calcium is able to be actively transported across the reticular membrane (table 4).

All purified fractions of membranes recovered from discontinuous sucrose gradients and isolated from muscle that had undergone either slow or rapid post-mortem decline in pH were able to actively accumulate calcium against a concentration gradient and hydrolyse ATP. The most active fragments from control muscle were recovered from fragments of intermediate bouyant density, N_2 ($1,158 < \rho < 1,184$ g/ml) which possessed a Ca-Binding capacity of 38,4 nmoles Ca^{2+}. mg protein^{-1} and, in the presence of oxalate accumulated calcium at the rate of 570 nmoles Ca^{2+}/min. mg protein^{-1} and hydrolysed ATP at the rate of 1,66 µmoles Pi/min. mg protein^{-1}. The binding capacity of the lightest material (N_3) ($\rho < 1,158$ g/ml) was comparable with that of N_2 but its rate of transport of calcium and rate of hydrolysis of ATP was diminished. Calcium accumulation of the dense band (N_1) ($1,184 < \rho < 1,210$ g/ml) was approximately one-half that of the other two bands although the ATPase activity was not significantly different.

The two purified fractions of membranes from PSE muscle displayed rates of calcium transport and of hydrolysis of ATP similar to one another. Capacity for calcium accumulation and rates of transport were approximately $^1/4$ those of the most active control membranes (N_2 and N_3) (13,0 versus 38,3 nmoles Ca^{2+}. mg protein^{-1} and 121,0 versus 510,9 nmoles Ca^{2+}/min. mg protein^{-1}). ATPase activity of the material of intermediate bouyant density (P_2) from PSE muscle had somewhat less ATPase activity than the

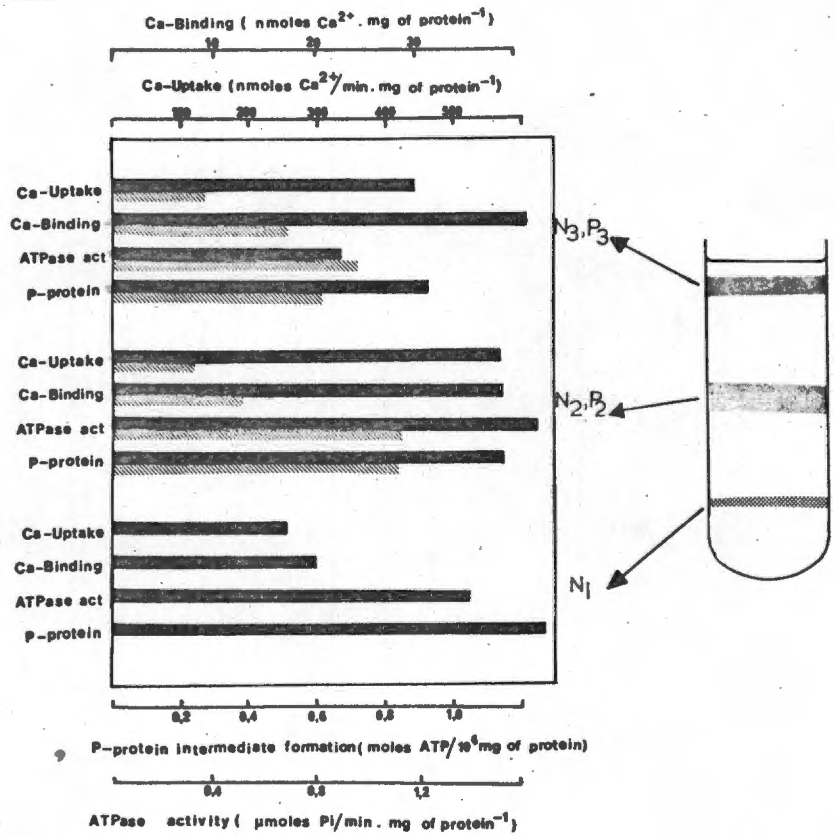


Fig. 14 The functional activities of fragmented sarcoplasmic reticulum.

The data are taken from table 4. FSR from slowly-glycolysing (■) and from PSE (▨) muscle.

corresponding normal fraction (1,13 versus 1,66 µmoles Pi/min. mg protein^{-1}) whilst that of the least dense fractions, N_3 and P_3 were closely similar (0,90 versus 0,97 µmoles Pi/min. mg protein^{-1}). The calcium-accumulating ability of the material from PSE muscle was therefore consistently diminished by comparison with all the purified fractions from slowly-glycolysing muscle whereas the ATPase activity was similar in magnitude.

The hydrolysis of ATP by both PSE and normal fragments proceeds via a phosphorylated intermediate (Table 4)

A summary of the data presented in this section is given in Fig.14 in the form of a histogram

3.3 PURIFICATION OF CRUDE MICROSOMAL FRACTIONS BY BOUYANT-DENSITY CENTRIFUGATION THROUGH A CONTINUOUS SUCROSE GRADIENT.

Summary: Bouyant density centrifugation through a continuous sucrose gradient (15–45% sucrose) of crude microsomal material isolated from slowly-glycolysing muscle results in a dense, aggregated band ($\rho > 1{,}200$ g/ml), a broad disperse band ($1{,}120 < \rho < 1{,}175$ g/ml) and a narrow band ($\rho = 1{,}100$ g/ml) of particulate material. The microsomal fractions from PSE muscle had an altered sedimentation pattern. Aggregated material sediments at $\rho = 1{,}175$ g/ml and is continuous with the disperse material above it ($1{,}120 < \rho < 1{,}175$g/ml). A narrow band is located at $\rho = 1{,}070$ g/ml and is surrounded by disperse material. It is apparent that part of the crude microsomal fraction from PSE muscle has a lower bouyant density than comparable material in control muscle. The absorption profile at 280 nm and protein determination on eluted fractions of the gradient confirm this conclusion and, in addition, reveal the presence of soluble proteins at the surface of the control gradient, which are absent from the PSE gradient. Ca^{2+}, Mg^{2+}-dependent ATPase activity (approx. 1,3 µmoles Pi/min. mg protein^{-1}) is greatest in the material banding in the central region of the gradients but the aggregated material in this particular gradient has the greater total activity due to the larger amount of protein. The disperse material from the PSE gradient has a higher specific activity (approx. 1,8 µmoles Pi/min. mg protein^{-1}) than that from the control gradient. The narrow bands from normal and from PSE gradients ($\rho = 1{,}100$ and 1070 g/ml respectively) contain no dectable ATPase activity.

Bouyant density centrifugation through a continuous sucrose gradient (15-45% $^{w}/_{v}$ sucrose) of the crude microsomal fraction isolated from slowly-glycolysing muscle resulted in the separation of three distinct membrane populations (fig 15). The material of greatest bouyant density sedimented to the bottom of the tube ($\rho > 1,200$ g/ml) and appeared fluffy and aggregated. It was distinct from a broad, disperse band in the central region of the gradient ($1,120 < \rho < 1,175$ g/ml). A third narrow band was visible near the top of the gradient ($\rho = 1,100$ g/ml). Similar treatment of the crude microsomal fraction from PSE muscle revealed altered sedimentation behavior. Virtually no material sedimented to the bottom of the tube but a fairly-sharp aggregated band of fluffy white material ($\rho = 1,175$ g/ml) appeared below the middle diffuse band. This aggregated material appears to correspond to the dense sediment from slowly-glycolysing muscle. Above ($1,120 < \rho < 1,175$ g/ml), the middle diffuse band corresponds to that in the control gradient. A small amount of material ($1,175 < \rho < 1,200$ g/ml), evident in slowly-glycolysing muscle, was absent from PSE microsomal fraction. A narrow band near the top of the tube corresponded to that in the control gradient except that its density had decreased ($\rho = 1,070$ g/ml <u>versus</u> 1,100 gm/ml). A small amount of disperse material was evident both above and below the narrow band in the PSE gradient. Thus all three populations of microsomal vesicles from PSE muscle exhibited a tendency towards decreased bouyant density. All the material in the lower, fluffy aggregate and in the upper, sharp band appeared to be affected. Most of the material in the central band ($1,120 < \rho < 1,175$ g/ml) was unchanged but the more

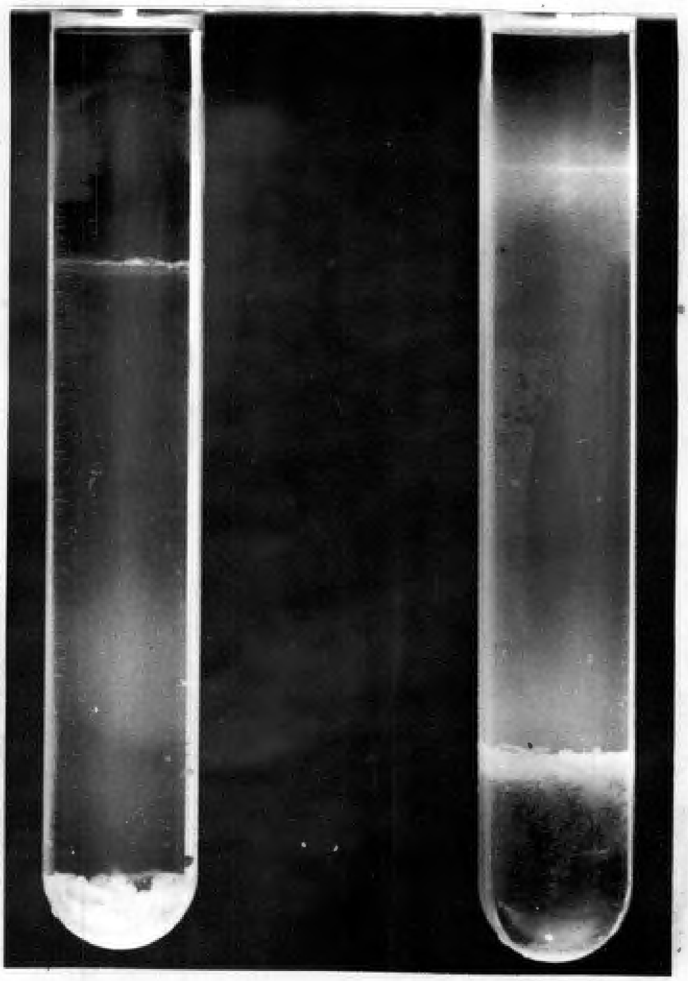


Fig. 15 Isopycnic sedimentation in a continuous sucrose gradient of a crude microsomal preparation isolated from slowly-glycolysing (normal) and from PSE muscle.

A crude microsomal fraction was isolated by differential centrifugation and suspended in 0,1M KCl, 5 mM histidine buffer, pH 7,4 (approx. 25 mg/ml). The suspension (1 ml) was layered on to a continuous sucrose gradient (15-45% sucrose) and centrifuged for 16 hrs at 60 000 xg in a swinging bucket rotor (Beckman SW 27.1).

dense material of this fraction was missing. The extra diffuse material sedimenting in the PSE gradient (1,070 $< \rho <$ 1,100 g/ml) may represent this population of vesicles.

The continuous gradient analysis was performed on a total of ten muscle samples and the banding pattern was reproducible.

In addition to visual examination, the gradients were analysed for protein. The gradient was eluted through a puncture in the bottom of the tube and monitored for UV absorbance at 280 nm (fig 16) and the fractions analysed by the Lowry method (fig 16c).

The elution profiles confirm visual assessment. The aggregated nature of the most dense material in both types of muscle is apparent from the erratic 280 nm absorbance. Paucity of UV absorbing material was evident at ρ = 1,183 g/ml in the PSE gradient. The sharp protein peak at ρ = 1,100 g/ml was absent from PSE microsomal material. A greater amount of material absorbing at 280 nm was present in the control profile in the lower density region (ρ = 1,100 g/ml). This material was not particulate and presumably represents soluble proteins or free tyrosine residues. A possible reason is that the PSE microsomal pellet, unlike that of the control, was compact and it was easier to remove traces of supernatant from its surface after sedimentation during differential centrifugation.

The arrows in fig 16a and 16b indicate the limits of the discontinuous gradients used in Section 3.2. The components of each band of the latter gradients can clearly be distinguished. The lower band, N_1, contained the dense, aggregated material, in addition to some of the reticulum from the broad, monodisperse band. The analogous band P_1 contained no protein

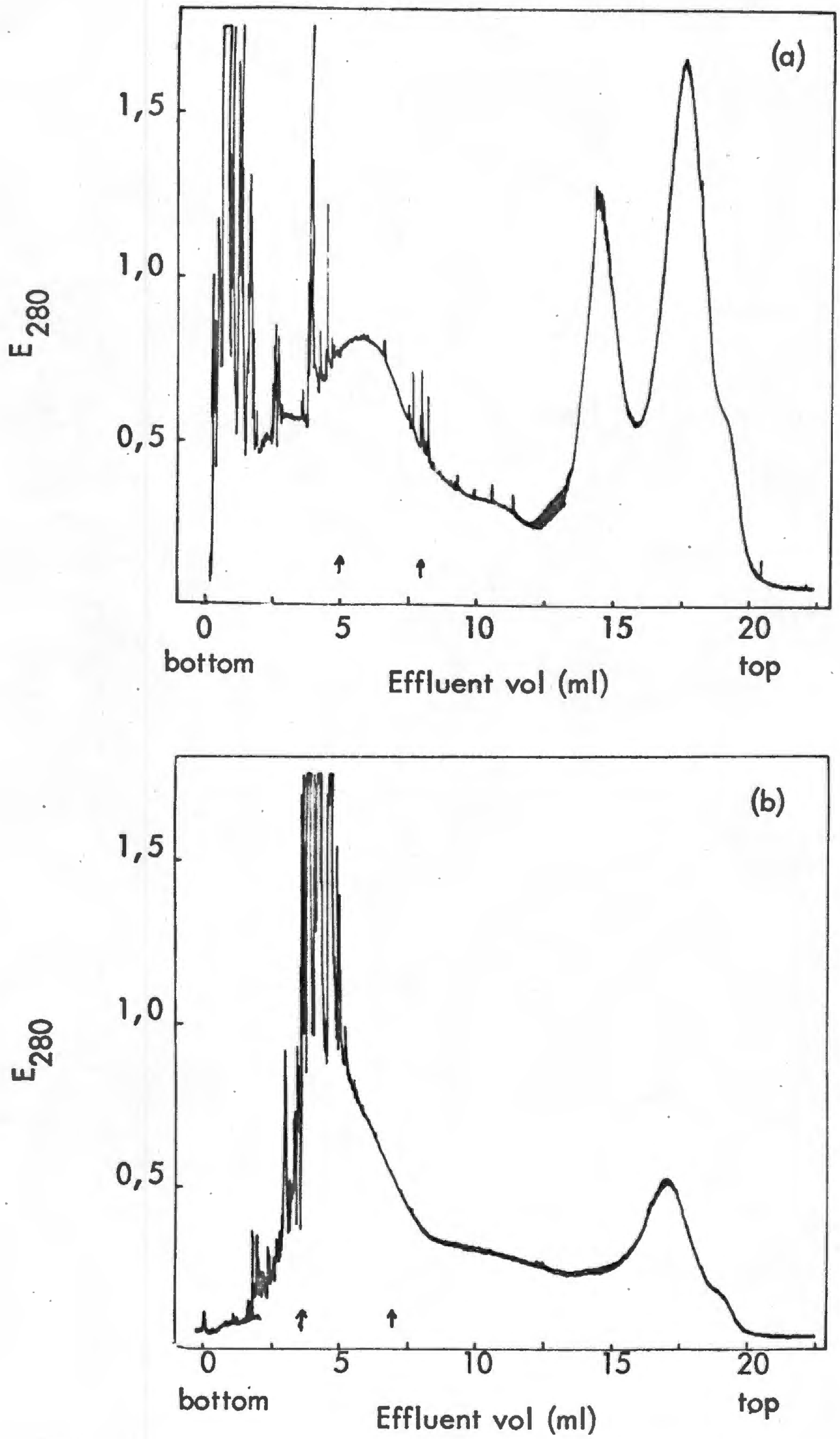


Fig. 16 Protein concentrations and ATPase activity of microsomal fractions from slowly-glycolysing and from PSE muscle sedimented in continuous sucrose gradients.

The gradient (see fig. 15) was eluted from a puncture in the bottom of the centrifuge tube by pumping in H_2O at the top of the tube at 0,8 ml/min. The eluate was passed through a flow cell positioned in a Unicam spectrophotometer and fractions (0,5 ml) were collected. (a) Absorbance at 280 nm of microsomal fraction from slowly-glycolysing muscle and (b) from PSE muscle.

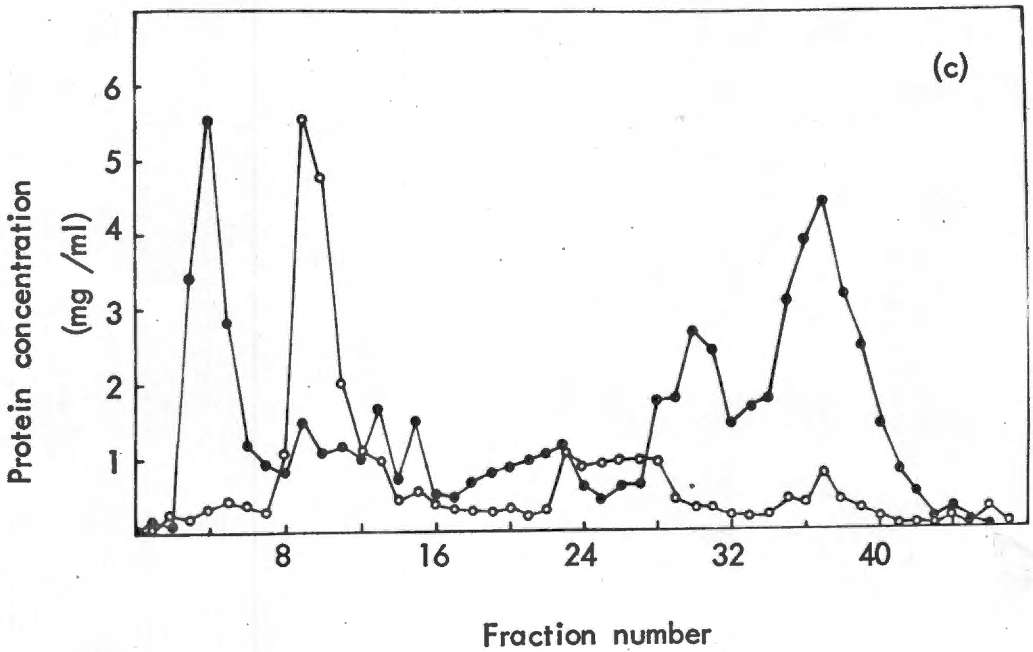
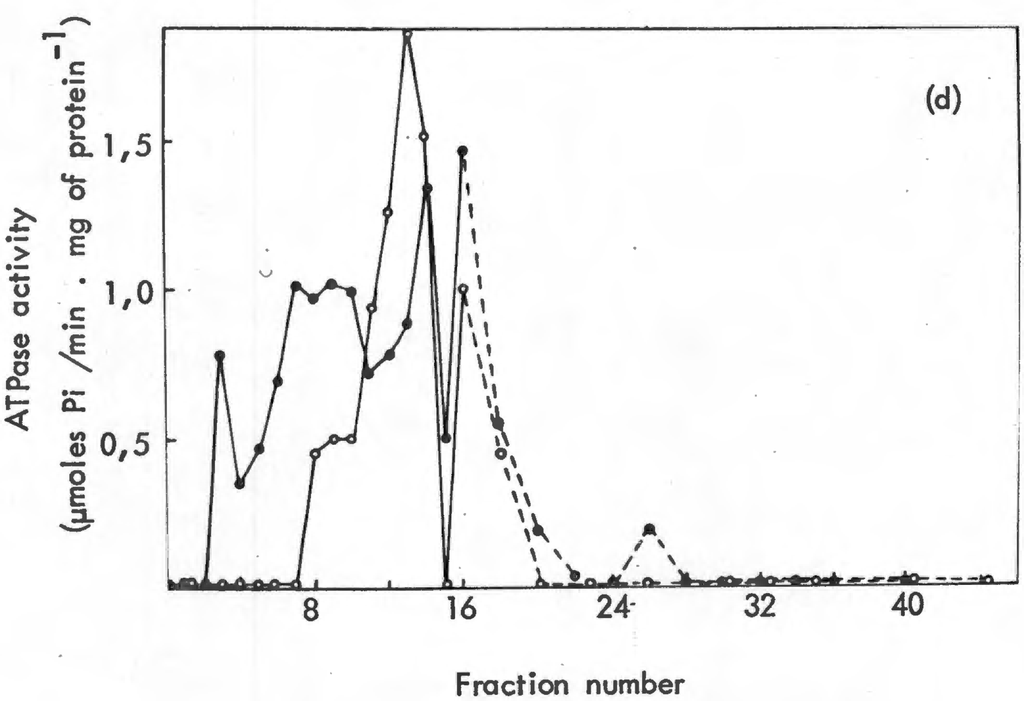


Fig. 16 Cont. (c) Protein concentration (Lowry method) of fractions (0,5 ml) eluted from a continuous sucrose gradient containing microsomal material from slowly-glycolysing muscle (•) and from PSE muscle (o).



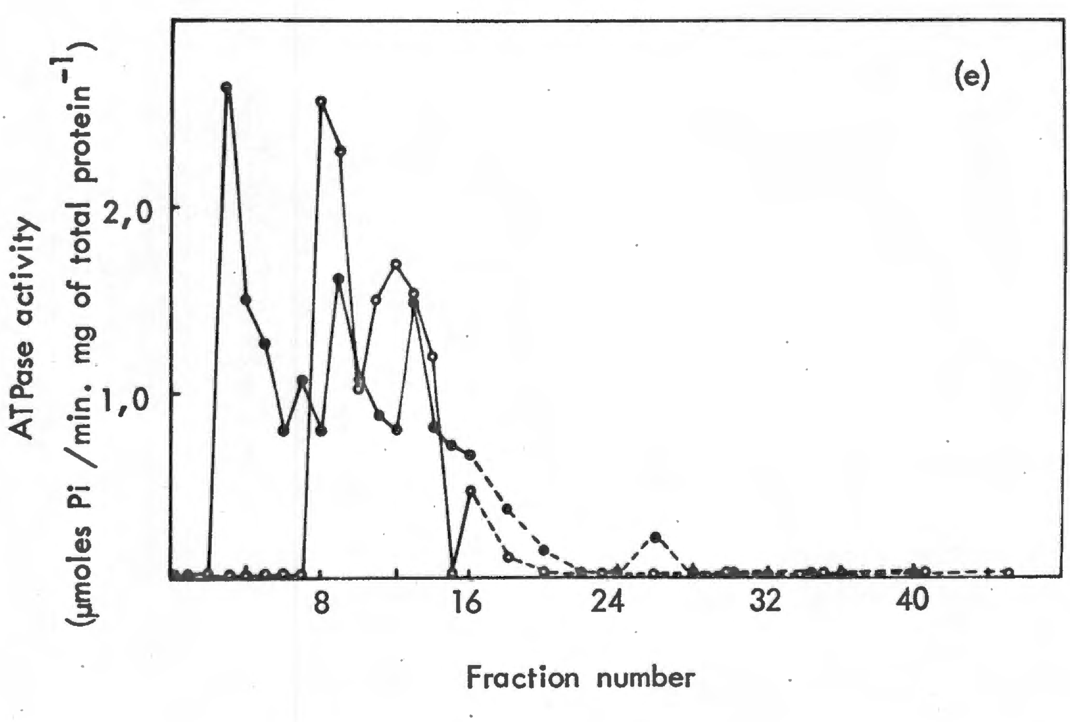


Fig. 16 cont. (d) ATPase activity expressed per mg protein, (e) Total ATPase activity per fraction. • control; o PSE.

material owing to migration of the dense band to a lower bouyant-density region. The middle band, N_2, is seen to be composed of a single population of vesicles whereas P_2 also contained the dense, aggregated material. The top bands N_3 and P_3 contained vesicles of the monodisperse middle band as well as those from the narrow band (ρ = 1,100 g/ml and 1,070 g/ml respectively) and the soluble proteins, which the normal gradient contained in greater amount.

Figure 16d shows the ATPase activity residing in each of the fractions eluted. All the fractions containing protein, from the highest bouyant-density of 1,200 g/ml to that of about 1,146 g/ml (approximately fraction 20) had the ability to hydrolyse ATP. ATPase of the dense, aggregated material had a lower specific activity than had the middle region (1,150 - 1,170 g/ml). No activity was detectable in the material banding above the 1,150 g/ml region. The total activity of crude microsomal material from the two muscle types was similar (fig 16e). The specific activity of the purified fractions was also comparable. These results are in agreement with those obtained with purified fractions from discontinuous gradients (fig 10).

3.5 FATTY ACID COMPOSITION OF LIPID EXTRACTS FROM WHOLE MUSCLE.

Summary: The most abundant fatty acids in porcine skeletal muscle lipid extracts are those of 16 and 18 carbon atoms in length. The phospholipid fraction contained predominantly palmitate (C16:0), stearate (C18:0), oleate (C18:1ω9) and linoleate (C18:2ω6) (in all, 82% of total fatty acids) and the neutral lipid fraction mainly palmitate and oleate (66% of total fatty acids). Phospholipids, in addition, contained significant amounts of long chain polyunsaturated fatty acids and fatty acids containing vinyl ether linkages. There were no gross differences between the fatty acid composition of slowly glycolysing (normal) muscle and PSE muscle, but small, significant decreases in the proportion of stearate, oleate and docosapentenoate (C22:5ω3) and an increase in linoleate were found in the phospholipid extracts of PSE muscle. These differences were largely due to alterations in the fatty acid composition of phosphatidyl choline, the major phospholipid class. The results are, in addition, summarised in the form of histograms in fig 18.

Lipid accounted for 3,4% of the total weight of slowly-glycolysing (normal) porcine muscle and of this 88,5% was neutral lipid. This was in contrast to PSE muscle which had one-half of the normal amount of extractable lipid and less than half of the neutral lipid content of control muscle (Table 5). The total phosphorus content of lipid extracts was not significantly different in the two muscle types.

Table 5. <u>Total extractable lipids from whole porcine skeletal muscle.</u>

Freshly excised tissue (1 g) was homogenised in 20 vols chloroform-methanol (2:1) and the lipids extracted according to the method of Folch <u>et al.</u> (1957) as described in 'Methods". Aliquots of 0,2 ml extract were pipetted into aluminium pans, evaporated to dryness and weighed on a Cahn microbalance (see 'Methods'). For phosphorus determination aliquots (containing 0,5 - 10 μg P) were dried at the bottom of a pyrex test-tube in a stream of N_2 and the residue digested with 72% perchlorate. Organic phosphorus was then reduced to phosphomolybdate and the absorbance of the blue colour measured at 830 nm. The neutral lipid content was obtained by subtraction. The results are the mean ± S.D. of 3 determinations.

	Total extractable lipid (mg/g wet muscle)	phospholipid (mg/g wet muscle)	neutral lipid (mg/g wet muscle)
NOR	34,35 ± 6,62	3,97 ± 0,39	30,38
PSE	17,26 ± 2,48	4,61 ± 0,55	12,65

The fatty acid composition of whole muscle lipid extracts was analysed by gas-liquid chromatography (table 6). The predominant fatty acids were palmitate (21,9%), stearate (11,9%), oleate (32,4%) and linoleate (22,9%). There were no significant differences between the relative amounts of each fatty acid derived from extracts of slow versus fast-glycolysing muscle. Significant quantities of the dimethyl acetal derivatives of palmitaldehyde and stearaldehyde were detected (4% and 1% respectively). The fatty acids of PSE and of slowly-glycolysing muscle showed a similar degree of unsaturation, as measured from the relative number of double bonds.

The total extractable lipids were separated on silicic acid columns into phospholipid (polar) and neutral (nonpolar) fractions. The phospholipids were characterised by the presence of long-chain, polyunsaturated fatty acids, vinyl ether linkages (as evidenced from dimethyl acetal derivatives) and the predominance of linolenate (28,3%). The neutral lipids exhibited a similar fatty acid composition to that of total lipids except that, with the phospholipids removed, the quantity of linolenate decreased from 22,9% to 10,1% and oleate increased from 32,4% to 44,9% of the total acids present. Polyunsaturated acyl chains were few in number.

The phospholipids of PSE pork differed from normal in showing a small but significant decrease in the relative proportion of stearate (16,0 to 14,0%), oleate (19,5 to 17,2%), and eicosapentenoate (2,30 to 1,63%) and an increase in linolenate (28,3 to 30,6%). The average degree of unsaturation, however, did not change (143 versus 146 relative number of double bonds). Neutral lipids from the two muscle types did not differ in their fatty acid composition.

Table 6 Fatty acid composition of total lipids from extracts of slowly-glycolysing (normal) and of PSE porcine muscle.

The fatty acid composition of lipid extract of whole muscle was determined by means of GLC of their methyl ester derivitives. Results (mean $\pm$ S.E.M, n=10) are expressed as a percentage of the area of the gas chromatographic peak compared to the total area of all measurable peaks. The relative number of double bonds was calculated as $\sum_{1}^{n}$ $(FA_n . DB_n)$ where FA_n is % of the total amount of fatty acids of the n th fatty acid and DB_n is the number of unsaturated double bonds in that fatty acid.

	Percentage Composition	
Fatty Acid	Normal	PSE
C14:0	0,88 $\pm$ 0,11	0,75 $\pm$ 0,09
C16:ald	3,9 $\pm$ 0,36	4,6 $\pm$ 0,51
C16:0	21,9 $\pm$ 0,50	21,3 $\pm$ 0,63
C16:1	2,75 $\pm$ 0,06	3,2 $\pm$ 0,19
C18:ald	1,34 $\pm$ 0,22	1,71 $\pm$ 0,26
C18:0	11,9 $\pm$ 1,3	13,9 $\pm$ 0,65
C18:1ω9	32,4 $\pm$ 1,8	28,9 $\pm$ 1,7
C18:2ω6	22,9 $\pm$ 1,4	23,1 $\pm$ 0,89
C20:4ω6	3,0 $\pm$ 0,39	3,5 $\pm$ 0,36
C20:5ω3	2,8 $\pm$ 0,51	2,4 $\pm$ 0,09
Relative Number of double bonds	107	103

Table 7 Fatty acid composition of the phospholipid fraction from extracts of slowly glycolysing (normal) and of PSE porcine muscle.

The percentage composition (mean $\pm$S.E.M., (n)) of fatty acids and relative number of double bonds were calculated as described in Table 6 Probability values were calculated by students t test.

Fatty Acid	Percentage Composition: Normal	PSE
C14:0	0,94 $\pm$ 0,22 (23)	1,04 $\pm$ 0,16 (23)
C16:ald	4,5 $\pm$ 0,4 (23)	3,7 $\pm$ 0,32 (23)
C16:0	18,4 $\pm$ 0,9 (23)	20,6 $\pm$ 1,0 (23)
C16:1	1,31 $\pm$ 0,10 (23)	1,2 $\pm$ 0,11 (23)
C18:ald	1,94 $\pm$ 0,32 (23)	1,5 $\pm$ 0,23 (23)
C18:0	16,0 $\pm$ 1,0 (23)	14,0 $\pm$ 0,89 (23)**
C18:1ω9	19,5 $\pm$ 1,0 (23)	17,2 $\pm$ 0,81 (23)
C18:2ω6	28,3 $\pm$ 1,0 (23)	30,6 $\pm$ 0,87 (23)**
C18:3ω3	$<$0,50 (6)	$<$0,50 (5)
C20:0	$<$0,50 (6)	$<$0,50 (5)
C18:4ω3	0,61 $\pm$ 0,05 (6)	0,52 $\pm$ 0,05 (5)
C20:2ω6	1,11 $\pm$ 0,06 (6)	1,28 $\pm$ 0,14 (5)
C22:0	$<$0,50 (6)	$<$0,50 (5)
C20:4ω6	4,9 $\pm$ 0,4 (23)	5,7 $\pm$ 0,52 (23)
C20:5ω3	2,8 $\pm$ 0,3 (23)	3,5 $\pm$ 0,61 (23)
C22:5ω3	2,30 $\pm$ 0,09 (6)	1,63 $\pm$ 0,10 (5)**
C24:2ω3	2,60 $\pm$ 0,20 (6)	2,00 $\pm$ 0,03 (5)
Relative Number of double bonds	143	146

** P $<$0,05

Table 8 Fatty acid composition of the neutral lipid fraction from extracts of slowly glycolysing (normal), and PSE porcine muscle.

The percentage composition (mean $\pm$ S.E.M, n=18) was calculated as described in Table 6.

	Percentage Composition	
Fatty Acids	Normal	PSE
C14:0	2,87 $\pm$ 0,39	2,73 $\pm$ 0,30
C16:ald	1,68 $\pm$ 0,39	3,31 $\pm$ 0,50
C16:0	21,0 $\pm$ 0,57	21,1 $\pm$ 0,64
C16:1	6,2 $\pm$ 0,32	5,7 $\pm$ 0,28
C18:ald	1,43 $\pm$ 0,41	2,40 $\pm$ 0,46
C18:0	11,4 $\pm$ 0,48	11,0 $\pm$ 0,48
C18:1ω9	44,9 $\pm$ 0,97	41,8 $\pm$ 1,30
C18:2ω6	10,1 $\pm$ 0,77	9,7 $\pm$ 0,78
C18:3ω3	$<$0,50	$<$ 0,50
C20:0	$<$0,50	$<$ 0,50
C18:4ω3	$<$ 0,50	$<$ 0,50
C20:2ω6	$<$0,50	$<$ 0,50
C22:0	$<$0,50	$<$ 0,50
C20:4ω6	$<$0,50	$<$ 0,50
C20:5ω3	$<$0,50	$<$ 0,50
C24:1	$<$0,50	$<$ 0,50
C22:5ω3	$<$0,50	$<$ 0,50
C24:2ω3	$<$0,50	$<$ 0,50

Thin-layer chromatography of the phospholipid fraction resolved it into four readily-discernible bands which were identified as lysophosphatidyl choline *plus* sphingomyelin, phosphatidyl choline,phosphatidyl inositol *plus* serine and phosphatidyl ethanolamine. Each phosphatide fraction was found to have a characteristic fatty acid profile (table 9)

Lysophosphatidyl choline and sphingomyelin were identifiable by the large amount of stearate (31,5%) and approximately equal quantities of palmitate, oleate and linoleate (18,8, 16,6, and 17,9% respectively). The proportion of arachidonic acid from PSE muscle was double that in slowly-glycolysing muscle (4,4 to 9,4%). The major class, phosphatidyl choline, was composed of equal quantities of palmitate and linoleate ($\pm$ 25%) and also of stearate and oleate ($\pm$ 18%). Stearate was found to be diminished (17,3 to 9,7%) and linoleate more abundant (25,0 to 31,9%) in PSE muscle. Oleate was lowered from 18,4 to 14,3% but the differences were not significant. Differences in composition seen in the phosphatidyl choline class are similar to those found for the total phospholipid mixture, which could be expected since phosphatidyl choline is the most abundant phospholipid. Phosphatidyl inositol *plus* serine contained mainly linoleate (42,3%) with characteristically small amounts of palmitate (14,5%), oleate (14,3%) and stearate (8,6%). Phosphatidyl ethanolamine was characterised by the presence of relatively large quantities of palmitaldehyde (5,7%) and stearaldehyde (5,7%) and low palmitate (5,3%) content. The acyl chains in this fraction consisted mainly of stearate (24,6%), linoleate (23,2%), oleate (10,4%) and arachidonate (9,2%). There were no significant alterations in the latter three classes in PSE muscle.

Table 9 Fatty acid composition of phospholipid classes from extracts of slowly-glycolysing (normal) and of PSE porcine muscle.

The percentage composition of fatty acids (mean $\pm$ S.E.M., n=5) was calculated as in table 6.

Fatty Acids	Percentage Composition: Lysophosphatidyl choline plus sphingomyelin		Percentage Composition: Phosphatidyl choline	
	Normal	PSE	Normal	PSE
C14:0	0,49 $\pm$ 0,16	0,94 $\pm$ 0,21	0,53 $\pm$ 0,39	0,36 $\pm$ 0,21
C16:ald	1,95 $\pm$ 0,53	0,79 $\pm$ 0,30	3,1 $\pm$ 2,52	1,23 $\pm$ 0,53
C16:0	18,8 $\pm$ 3,0	12,1 $\pm$ 2,0	24,7 $\pm$ 3,9	31,7 $\pm$ 4,12
C16:1	0,88 $\pm$ 0,25	1,26 $\pm$ 0,30	0,86 $\pm$ 0,20	0,09 $\pm$ 0,29
C18:ald	0,45 $\pm$ 0,17	0,12 $\pm$ 0,09	0,50 $\pm$ 0,26	0,25 $\pm$ 0,10
C18:0	31,5 $\pm$ 4,4	29,7 $\pm$ 5,1	17,3 $\pm$ 2,53	9,7 $\pm$ 1,65**
C18:1ω9	16,6 $\pm$ 2,8	12,7 $\pm$ 1,3	18,4 $\pm$ 2,34	14,3 $\pm$ 0,82
C18:2ω6	17,9 $\pm$ 3,6	21,5 $\pm$ 2,5	25,0 $\pm$ 1,71	31,9 $\pm$ 1,04**
C20:4ω6	4,4 $\pm$ 1,71	9,4 $\pm$ 0,69**	2,91 $\pm$ 1,02	5,1 $\pm$ 1,71
C20:5ω3	1,0 $\pm$ 0,49	3,1 $\pm$ 1,05	1,10 $\pm$ 0,55	2,32 $\pm$ 0,95
Relative Number of double bonds	76	110	86	111

** $P < 0,05$

Table 9 cont.

Fatty Acid	Percentage Composition: Phosphatidyl Serine plus Phosphatidyl Inositol		Percentage Composition: Phosphatidyl Ethanolamine	
	Normal	PSE	Normal	PSE
C14:0	3,1 ± 1,14	3,34 ± 1,84	0,77 ± 0,16	0,92 ± 0,39
C16:ald	1,83 ± 0,70	1,60 ± 0,99	5,7 ± 4,6	2,80 ± 1,14
C16:0	14,5 ± 4,08	12,6 ± 3,3	5,3 ± 0,80	10,3 ± 2,99
C16:1	2,34 ± 0,71	3,4 ± 1,01	1,04 ± 0,24	1,14 ± 0,52
C18:ald	1,44 ± 0,29	1,89 ± 0,50	5,7 ± 0,53	2,15 ± 1,23
C18:0	8,6 ± 1,96	7,4 ± 1,37	24,6 ± 4,2	24,3 ± 3,6
C18:1 ω9	14,3 ± 1,06	12,5 ± 1,50	10,4 ± 1,05	11,1 ± 1,76
C18:2 ω6	42,3 ± 7,0	34,6 ± 8,2	23,2 ± 1,31	22,0 ± 0,96
C20:4 ω6	0,90 ± 0,56	2,51 ± 0,66	9,1 ± 1,89	11,6 ± 1,93
C20:5 ω3	0,47 ± 0,42	4,2 ± 2,34	3,8 ± 1,26	6,0 ± 1,69
Relative Number of double bonds	107	116	113	133

Each class of phospholipid from PSE muscle had a slightly greater degree of unsaturation in the acyl side chains.

The triglyceride fraction has a fatty acid profile very similar to that of the total neutral fraction, ie domination by oleate (43,3%) and few polyunsaturated acids detectable. The free fatty acids display a greater variety, with C24:1 assuming importance (8,8%). A significant elevation in linoleate (8,1 to 14,0%) was found in PSE muscle free fatty acids.

Table 10 Fatty acid composition of neutral lipid classes from extracts of normal and of PSE porcine muscle.

The percentage composition of fatty acids (mean $\pm$ S.E.M., n=5) was calculated as in table 6.

Fatty Acids	Percentage Composition			
	Triglycerides		Free Fatty Acids	
	Normal	PSE	Normal	PSE
C14:0	1,46 $\pm$ 0,45	1,76 $\pm$ 0,50	1,59 $\pm$ 0,95	1,59 $\pm$ 0,59
C16:0	22,0 $\pm$ 1,86	22,4 $\pm$ 2,20	16,2 $\pm$ 3,04	17,6 $\pm$ 1,67
C16:1	4,7 $\pm$ 0,79	5,8 $\pm$ 0,50	2,20 $\pm$ 0,34	1,95 $\pm$ 0,34
C18:ald	$<$0,50	$<$0,50	0,88 $\pm$ 0,04	0,70 $\pm$ 0,12
C18:0	18,7 $\pm$ 5,7	11,3 $\pm$ 0,83	16,6 $\pm$ 1,50	18,0 $\pm$ 0,61
C18:1ω9	43,3 $\pm$ 6,3	47,6 $\pm$ 2,23	30,0 $\pm$ 2,50	27,2 $\pm$ 1,14
C18:2ω6	8,2 $\pm$ 1,30	8,2 $\pm$ 1,57	8,1 $\pm$ 1,72	14,0 $\pm$ 1,03**
C20:4ω6	$<$0,50	$<$0,50	2,47 $\pm$ 0,53	3,11 $\pm$ 0,63
C20:5ω3	$<$0,50	$<$0,50	0,80 $\pm$ 0,34	0,76 $\pm$ 0,22
C22:5ω3	$<$0,50	$<$0,50	2,47 $\pm$ 1,57	1,23 $\pm$ 0,07
C24:1	$<$0,50	$<$0,50	8,8 $\pm$ 4,2	5,5 $\pm$ 2,50
C24:2	$<$0,50	$<$0,50	2,87 $\pm$ 1,40	1,94 $\pm$ 0,34

** P $<$0,05

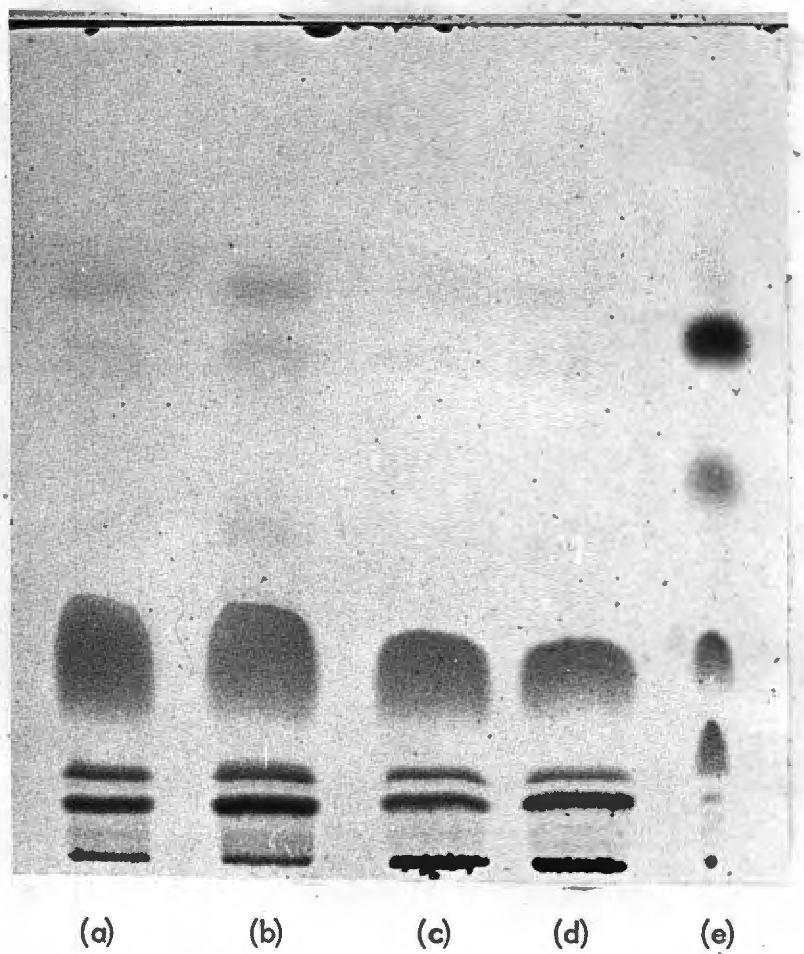


Fig. 17 Separation of neutral lipids from extracts of whole muscle by thin-layer chromatography.

Lipid extracts of whole muscle from slowly-glycolysing muscle (a and c) and from PSE muscle (b and d) were freed of phospholipids by silicic acid chromatography (a and b) and then chromatographed on the thin-layer plate and also chromatographed directly without such a purification step (c and d). Separation was achieved with silica gel G and a mixture of diethyl ether, acetic acid, petroleum ether (b.p. 60–80°C) (90:10:1) as solvent. Standards (e) consisted of oleate, triolein, methyl oleate and cholesterol oleate in order of increasing Rf values. The slowest moving band was identified as cholesterol.

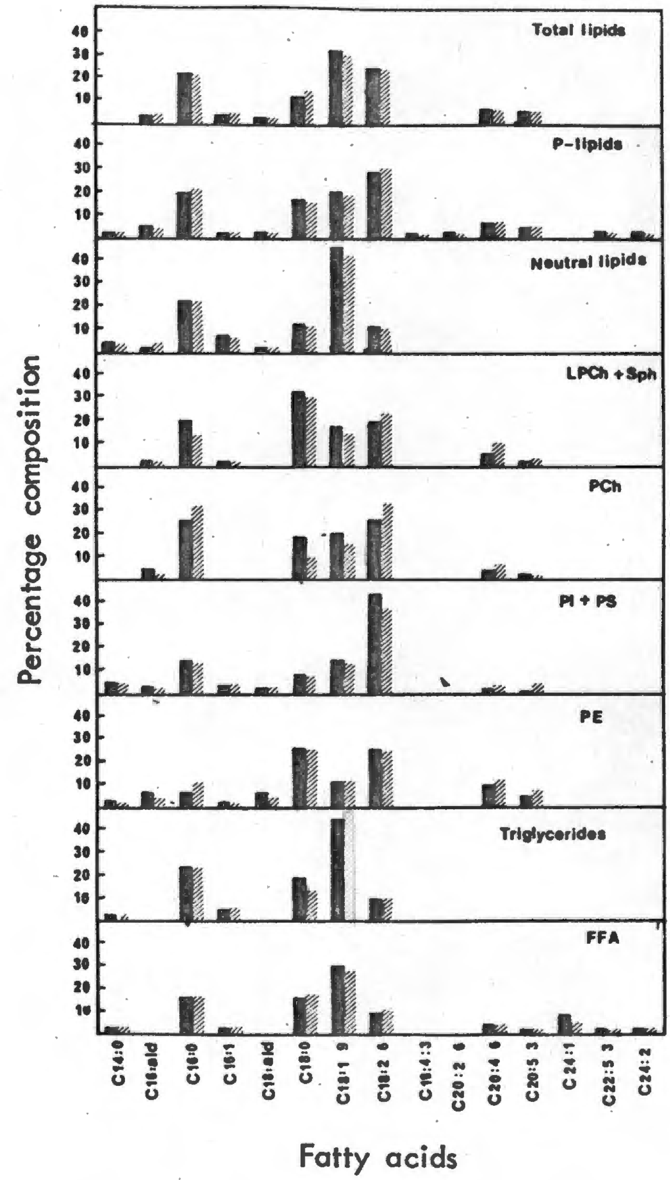


Fig. 18 The fatty acid composition of porcine whole muscle lipids.

The histograms represent the data in tables 6, 7, 8, 9, and 10. Extracts from slowly-glycolysing (■) and PSE (▨) muscle.

3.6 THE LIPID COMPOSITION OF FRAGMENTED SARCOPLASMIC RETICULUM.

Summary: Isolated membranes of fragmented sarcoplasmic reticulum (FSR) contain 0,30 mg of phospholipid and 0,050 mg of cholesterol per ,mg of protein. FSR isolated from rapidly-glycolysing, post-mortem muscle which showed pale, soft exudative (PSE) change had lower bouyant density on isopycnic sucrose density gradient centrifugation and increased ratios of lipid/protein. The composition of the phospholipid fraction of PSE membranes was similar to that of normal slowly-glycolysing muscle. In both, phosphatidyl inositol admixed with phosphatidyl serine and sphingomyelin occurring in decreasing concentrations. In membranes of low bouyant density ($\rho < 1,154$ g/ml), there was a small but significant enrichment in phosphatidyl choline of PSE when compared with slowly-glycolysing muscle, from 46,4% to 56,7%. Each phospholipid class had a characteristic fatty acid composition. Phosphatidyl choline was characterised by a large proportion of linoleate and palmitate (64%), phosphatidyl ethanolamine by a large quantity of aldehydes (22%), phosphatidyl inositol plus serine by the proportion of stearate and oleate (51%) and sphingomyelin by the preponderance of saturated fatty acids (50%) and tetracosenoate (34%). The only difference between PSE and slowly-glycolysing muscle was found in the phosphatidyl ethanolamine class where arachidonic acid was slightly but significantly decreased from 15,4% to 12,5% in PSE membranes.

Three fractions of FSR were isolated from slowly-glycolysing muscle N_1, N_2 and N_3 of decreasing densities 1,184-1,210 g/ml, 1,154-1,184 g/ml and 1,154 g/ml and these contained respectively 0,27, 0,31 and 0,34 mg phospholipid per mg of protein. The material from PSE muscle (P_2), having similar bouyant density to N_2 had a similar phospholipid content (0,31 versus 0,31 mg phospholipid per mg protein). The least dense material ($\rho < 1,158$ g/ml) from PSE muscle (P_3), which did not sediment, had significantly greater amounts of phospholipid than the corresponding material in N_3 (0,50 versus 0,34 mg phospholipid per mg protein).

Material of lowest bouyant density (N_3 and P_3) contained more cholesterol per mg protein than the denser material both in normal and in PSE membranes. In addition, P_3 contained significantly more cholesterol than did N_3 (0,097 versus 0,066 mg cholesterol per mg protein).

The results of lipid analysis of the membrane fractions from both muscle types can thus be correlated with the isopycnic sedimentation of membranes during discontinuous sucrose density centrifugation and indicate raised lipid/protein ratios in PSE membranes. This conclusion is based upon (i) the virtual absence of fraction P_1 from crude microsomal fractions of PSE muscle (fig 10) (ii) the increased amount of low-density material ($\rho < 1,158$ g/ml) in P_3 (table 2) (iii) the increased quantities of phospholipid and of cholesterol relative to protein in P_3 (table 10) and (iv) increase in absolute amounts of protein and of phospholipid in P_3 (deduced from protein recovered and lipid content per mg protein).

Table 11 Protein recovery and phospholipid and cholesterol content of FSR of varying bouyant density isolated from slowly-glycolysing muscle and from PSE porcine muscle.

Protein was determined by the Biuret method. Cholesterol and phospholipid were determined on $CHCl_3$–MeOH extracts of purified FSR. Phospholipid content was calculated from lipid-P assumming an average mol. wt. of phospholipids of 600. Results represent mean $\pm$SD (n).

Fraction	Protein Recovery (mg/g wet wt. muscle)	Phospholipid (mg/mg of protein)		Cholesterol (mg/mg of protein)	
N_3	0,16 $\pm$ 0,10 (14)	0,34 $\pm$ 0,02 (5)		0,066 $\pm$ 0,013 (5)	
P_3	0,32 $\pm$ 0,15 (14)	0,50 $\pm$ 0,01 (3)	**	0,097 $\pm$ 0,016 (6)	**
N_2	0,26 $\pm$ 0,17 (14)	0,31 $\pm$ 0,03 (5)		0,042 $\pm$ 0,012 (7)	
P_2	0,19 $\pm$ 0,16 (14)	0,31 $\pm$ 0,02 (5)		0,057 $\pm$ 0,016 (7)	
N_1	0,14 $\pm$ 0,09 (14)	0,27 $\pm$ 0,02 (2)		0,045 $\pm$ 0,008 (3)	
P_1	0,01	-		-	

$N_1 + N_2 + N_3$	0,56
$P_2 + P_3$	0,51

** P $<$0,01

The composition of lipid of membranes isolated from PSE muscle, which had relatively high lipid/protein ratios as compared with slowly-glycolysing muscle, was examined. The relative proportion of phosphatides was determined by TLC of extracts of polar phospholipids of the various purified fractions of FSR and quantitative phosphorus analysis of the separated phospholipids (see table 12).

The predominant phospholipid in all fractions was phosphatidyl choline (PCh), accounting for approximately 45-60% of total phospholipid. The highest concentration (60,1%) occurred in fraction N_2 and the lowest (46,4%) in N_3, whereas the concentration of this phosphatide in the corresponding fractions P_2 and P_3 (55,9% and 56,7% respectively) of PSE muscle were similar. The PCh content of P_3 (56,7%) was significantly higher than that of N_3 (46,4%). This finding suggests that the PCh-rich fraction, which in control FSR appears in N_2 ($1,158 < \rho < 1,184$ g/ml), has shifted into P_3 ($\rho < 1,158$ g/ml) due to lower bouyant density of the membranes, as a result of their increased lipid/protein ratio. The proportion of phosphatidyl ethanolamine was relatively constant in each band, at about 20% of the total. Phosphatidyl inositol and phosphatidyl serine were inseparable with the solvent system used and together made up approximately 11% of total phospholipids, although N_3 contained 15%. The quantity of sphingmyelin varied from 5% in N_2 to 12% of the total phospholipids in N_1 and N_3. Lysophosphatidyl choline was absent except for a small amount (2,6%) detected in N_1. The solvent front which carried the relatively nonpolar lipid series revealed a significant amount of phosphorus but the type of phospholipid (s) was not identified. Phosphatidyl ethanolamine was the most mobile phospholipid identified but was well separated from the solvent front and was unlikely to have contaminated the solvent front.

The fatty acid composition of the phosphatide species separated by TLC from purified FSR membranes isolated from slowly-glycolysing muscle and from PSE muscle, were determined by GLC analysis. The analysis was performed on the enriched preparations N_2 and P_2, which have been shown to contain the most functionally-active membranes isolated from the two muscle types. Each phospholipid class possessed a characteristic profile of aliphatic side chains, which included a large proportion of long-chain polyunsaturated fatty acids.

The most common aliphatic chains in the most abundant phospholipid class, phosphatidyl choline, are linoleate (34,2%) and palmitate (30,2%) which together make up approximately 64% of the total fatty acids found in this species. The remainder consists mainly of oleate, stearate and the polyunsaturated acids such as arachidonate. A significant amount of the dimethyl acetal derivative of palmitaldehyde was present in the elution profile indicating the presence of alk-1-enyl linkages of plasmalogens. The fatty acid profiles from the two muscle types were not significantly different. The phosphatidyl ethanolamine fraction was characterised by an unusually large amount of dimethyl acetal derivatives (totalling approx. 21%) and methyl esters of polyunsaturated fatty acids (approx. 50%). Three aldehydes, namely, palmitaldehyde (7,7%), stearaldehyde (8,7%) and olealdehyde (4,9%) were detected. The polyunsaturated fatty acids consisted predominantly of arachidonic acid (14,5%), linoleic acid (11,6%) and eicosapentenoic acid (18,1%). The fatty acid profile of this phosphatide from PSE muscle showed a significant decrease in arachidonic acid (14,5% <u>versus</u> 11,6% in control muscle). Combined phospholipids, phosphatidyl

Table 12 <u>The relative proportion of phospholipid classes in polar lipid extracts of FSR of varying bouyant density isolated from slowly glycolysing and from PSE muscle.</u>

The purified FSR fractions N_1, N_2, N_3, P_2 and P_3 were prepared as described in methods with discontinuous sucrose gradients (page 58). The amount of material in P_1 was neglible and too small for further analysis. $CHCl_3$-MeOH extracts of purified fractions were separated into phospholipid classes by TLC on silica gel-H by development in $CHCl_3$: MeOH: CH_3COOH: H_2O (25:15:4:2). Spots on TLC plate were visualised and analysed for P as described in "Methods". The results are expressed as % mean $\pm$ SD of total P recovered. The % recovery was calculated from the total P recovered compared to the amount of P applied to the plates. The material in the solvent front was not identified but represents the least polar phospholipids.

Phospholipids	Percentage Composition of Purified FSR Fractions				
	N_1	N_2	P_2	N_3	P_3
	n=1	n=5	n=5	n=2	n=2
Solvent front	8,2	4,0 $\pm$ 3,2	4,5 $\pm$ 1,8	3,3 $\pm$ 1,7	1,4 $\pm$ 2,0
Phosphatidyl Ethanolamine	17,5	19,2 $\pm$ 5,3	21,1 $\pm$ 1,9	18,6 $\pm$ 6,0	22,0 $\pm$ 0,95
Phosphatidyl Inositol + Phosphatidyl Serine	10,9	11,0 $\pm$ 1,5	10,9 $\pm$ 1,0	15,5 $\pm$ 2,8	10,2 $\pm$ 2,2
Phosphatidyl Choline	48,6	60,1 $\pm$ 6,2	55,9 $\pm$ 3,5	46,4 $\pm$ 1,1	56,7 $\pm$ 1,8**
Sphingomyelin	11,6	5,0 $\pm$ 1,7	6,3 $\pm$ 1,4	11,9 $\pm$ 5,3	14,0 $\pm$ 2,3
Lysophophatidyl Choline	2,6	$<$1,0	$<$1,0	$<$1,0	$<$1,0
Origin	$<$1,0	$<$1,0	$<$1,0	$<$1,0	$<$1,0
% Recovery	91,2	90,0 $\pm$ 8,0	84,0 $\pm$ 11,3	88,0 $\pm$ 4,5	84,1 $\pm$ 13,1

** P $<$ 0,05

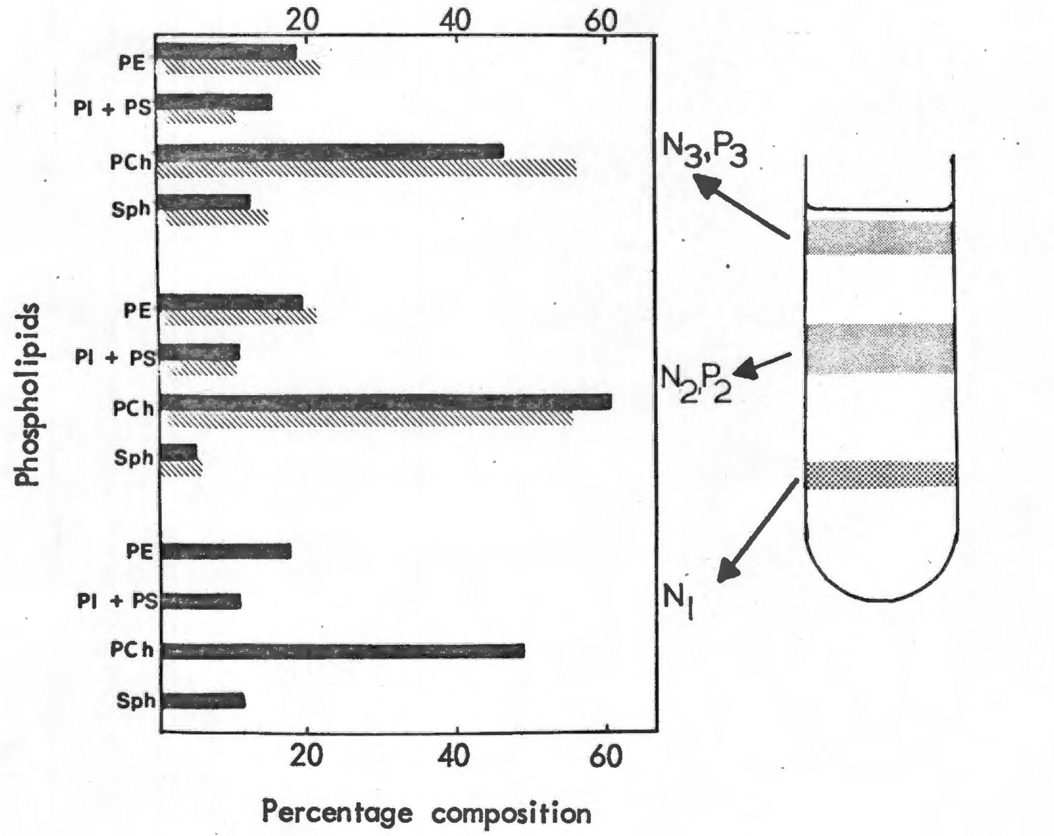


Fig. 19 Phospholipid composition of fragmented sarcoplasmic reticulum.

The data are taken from table 12 . FSR from slowly-glycolysing (■) and from PSE (▨) muscle.

Table 13 Fatty acid composition of phospholipid classes from extracts of FSR isolated from slowly glycolysing (normal) and from PSE muscle.

Lipid extracts of purified FSR fractions, N_2 and P_2 were separated into individual phospholipid components by TLC (see "Methods"). Each phospholipid fraction was eluted from the silica gel by a series of solvents of varying polarity, dried and methylated. The fatty acid methyl esters were extracted and separated by GLC using 10% DEGA as stationary phase at 188°C. The area under each peak was calculated as the product of peak height and width at half peak height. The results are expressed as a percentage (mean ± S.E.M., n=8) of the area of the gas chromatographic peak compared to the total area of all measurable peaks.

Fatty Acids	Percentage Composition			
	Sphingomyelin		Phosphatidyl Choline	
	Normal	PSE	Normal	PSE
C16:ald	<0,50	<0,50	3,1 ± 1,4	3,5 ± 0,21
C16:0	9,5 ± 1,6	13,8 ± 1,8	30,2 ± 2,5	28,0 ± 2,2
C16:1	1,7 ± 0,99	1,5 ± 0,59	<0,50	<0,50
C18:0	12,3 ± 1,6	12,9 ± 4,0	5,9 ± 0,38	7,0 ± 0,51
C18:1 ω 9	12,9 ± 1,5	15,4 ± 1,9	15,5 ± 0,93	17,9 ± 0,96
C18:2 ω 6	2,6 ± 0,36	4,8 ± 2,1	34,2 ± 1,1	34,8 ± 1,51
C20:0	14,3 ± 1,1	10,5 ± 1,3	<0,50	<0,50
C22:0	9,8 ± 0,39	9,4 ± 1,7	<0,50	<0,50
C20:3 ω 3	<0,50	<0,50	1,2 ± 0,20	1,8 ± 0,23
C20:4 ω 6	<0,50	<0,50	3,2 ± 0,17	3,1 ± 0,51
C20:5 ω 3	<0,50	<0,50	2,5 ± 0,17	2,5 ± 0,41
C24:0	3,1 ± 0,35	2,0 ± 0,84	<0,50	<0,50
C24:1 ω ?	34,0 ± 2,3	30,4 ± 5,5	<0,50	<0,50
C22:5 ω 3	<0,50	<0,50	1,23 ± 0,16	2,1 ± 0,60
C24:2 ω?	<0,50	<0,50	1,18 ± 0,2	2,0 ± 0,72

Table 13 cont.

Fatty Acids	Percentage Composition: Phosphatidyl Ethanolamine, Normal	Phosphatidyl Ethanolamine, PSE	Phosphatidyl Inositol + Phosphatidyl Serine, Normal	Phosphatidyl Inositol + Phosphatidyl Serine, PSE
C16:ald	7,7 ± 1,8	6,9 ± 1,0	<0,05	<0,05
C16:0	5,1 ± 0,77	5,4 ± 1,0	9,3 ± 1,67	11,4 ± 1,98
C16:1	1,6 ± 0,61	0,51 ± 0,21	2,3 ± 0,76	2,7 ± 0,18
C18:ald	8,7 ± 0,84	8,8 ± 0,65	<0,50	<0,50
C18:1ald	4,9 ± 0,68	7,3 ± 1,4	<0,50	<0,50
C18:0	15,3 ± 2,2	16,1 ± 2,0	36,3 ± 2,5	34,0 ± 2,8
C18:1 ω9	9,9 ± 1,0	9,9 ± 0,85	17,2 ± 1,49	16,6 ± 0,94
C18:2 ω6	11,6 ± 1,1	13,5 ± 1,24	9,8 ± 0,49	10,6 ± 1,03
C18:3 ω3	0,53 ± 0,09	0,66 ± 0,13	2,5 ± 1,18	2,1 ± 0,21
C20:0	<0,50	<0,50	2,4 ± 1,21	3,7 ± 0,67
C20:1 ω9	0,76 ± 0,35	<0,50	4,1 ± 0,53	4,0 ± 0,69
C20:3 ω3	2,50 ± 0,28	2,62 ± 0,31	<0,50	<0,50
C20:4 ω6	14,5 ± 1,03	11,6 ± 0,52**	12,1 ± 1,25	10,3 ± 1,56
C20:5 ω3	8,1 ± 0,70	8,1 ± 0,71	4,4 ± 1,72	2,8 ± 0,43
C22:3 ω3	1,18 ± 0,32	0,91 ± 0,56	<0,50	<0,50
C22:5 ω3	5,7 ± 0,70	4,3 ± 0,82	2,4 ± 0,05	2,5 ± 0,55
C24:2	4,8 ± 0,47	5,1 ± 1,3	1,6 ± 0,24	3,6 ± 1,00**

** P <0,05

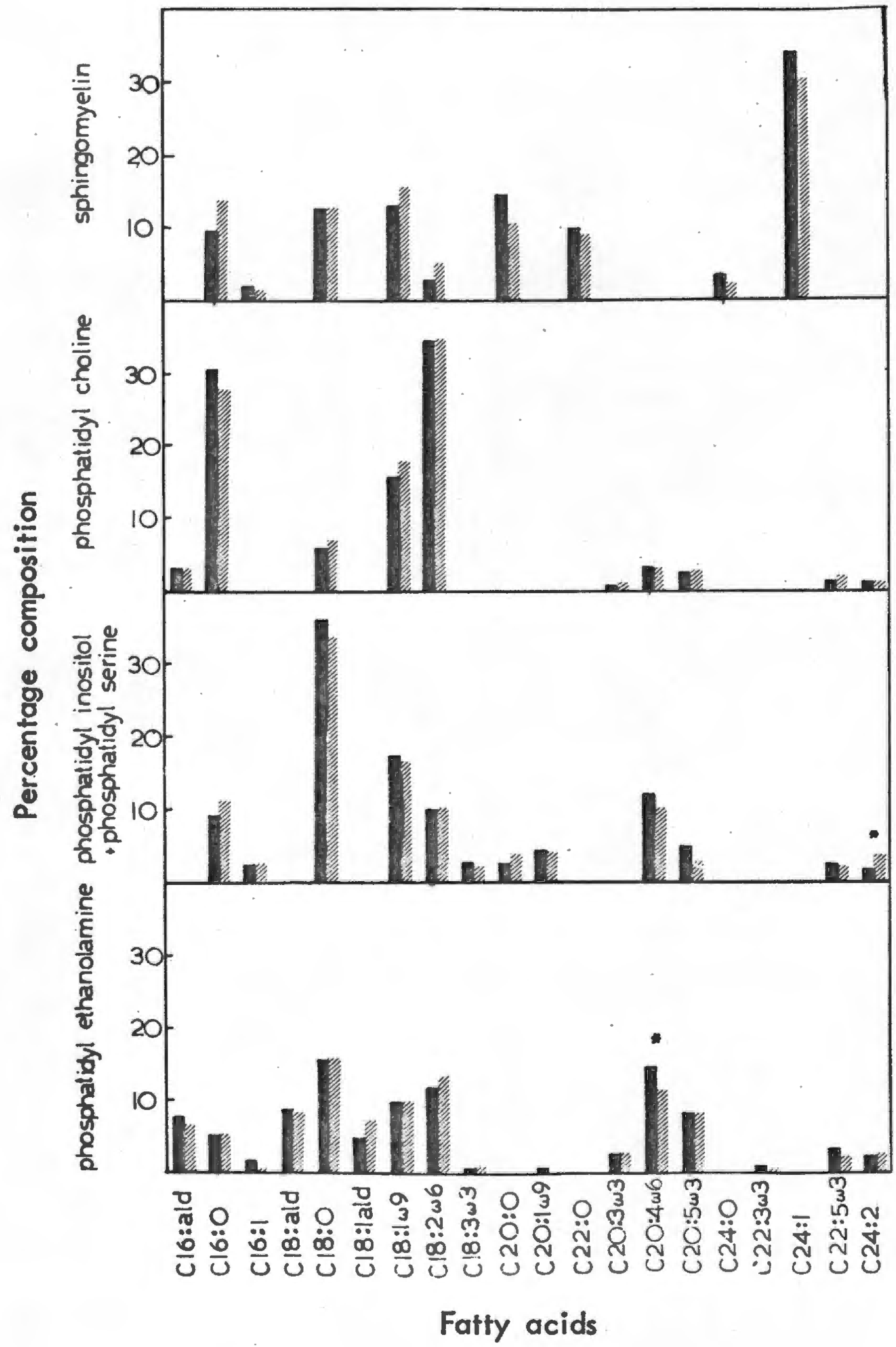


Fig. 20 <u>The fatty acid composition of phospholipids extracted from fragmented sarcoplasmic reticulum.</u>

The histograms represent the data in Table 12. FSR isolated from slowly-glycolysing muscle (■) and from PSE muscle (▨). (*) denotes a significant difference ($P < 0,05$).

inositol and phosphatidyl serine were largely made up of stearate (36,3%), oleate (17,2%), linoleate (9,8%) and arachidonate (12,1%). Dimethyl acetal derivatives were not detectable. Tetracosadienate (C24:2), was significantly raised in PSE muscle (3,6% versus 1,6% in control muscle). Aliphatic side chains of sphingomyelin were unusual in that saturated fatty acids predominated and tetracosenoate (C24:1) was present in large quantities. The saturated fatty acids consisted of palmitate (9,5%), stearate (12,3%), eicosanoate (14,3%), docosanoate (9,8%) and tetracosanoate (3,1%). Tetracosenoate constituted 34,0% of the total acyl chains. Oleate was the only other unsaturated acid present in significant amounts (12,9%).

3.7 PROTEIN COMPOSITION OF FRAGMENTED SARCOPLASMIC RETICULUM.

Summary: At least eleven protein components can be distinguished in FSR membranes of porcine skeletal muscle. A similar number of components is present in membranes from rabbit skeletal muscle. The predominant band is the ATPase protein (mol. wt. 105 000) followed by four or five bands containing approximately equal amounts of protein. These may include calsequestrin (46 5000) and a high affinity calcium-binding protein (55 000) present in rabbit FSR. No consistent qualitative or quantitative difference could be detected between protein composition of FSR of slowly-glycolysing muscle and of PSE muscle.

The protein components of FSR membranes of slowly- and of rapidly-glycolysing muscle were examined by sodium dodecyl sulphate (SDS) polyacrylamide gel disc electrophoresis. Membranes were solubilised in 1% SDS prior to electrophoresis in 0,1% SDS which was included in the gel and reservoir buffers. The stained gels were examined visually (fig 21) and by scanning densitometry (fig 22). Normal porcine FSR contained several discrete proteins. The major fraction corresponds to the 105 000 mol. wt. Ca^{2+}-ATPase or calcium pump protein (Martonosi and Halpin, 1971). Other proteins of molecular weights 47 000 and 54 000 are present, which could correspond to calsequestrin (46 500) and a high-affinity calcium-binding protein (55 000) described by Ostwald and MacLennan (1974). Other bands in the region 55 000 to 100 000 mol. wt. and 15 000 to 40 000 correspond to similar bands in rabbit muscle. A stained gel of the proteins of FSR from

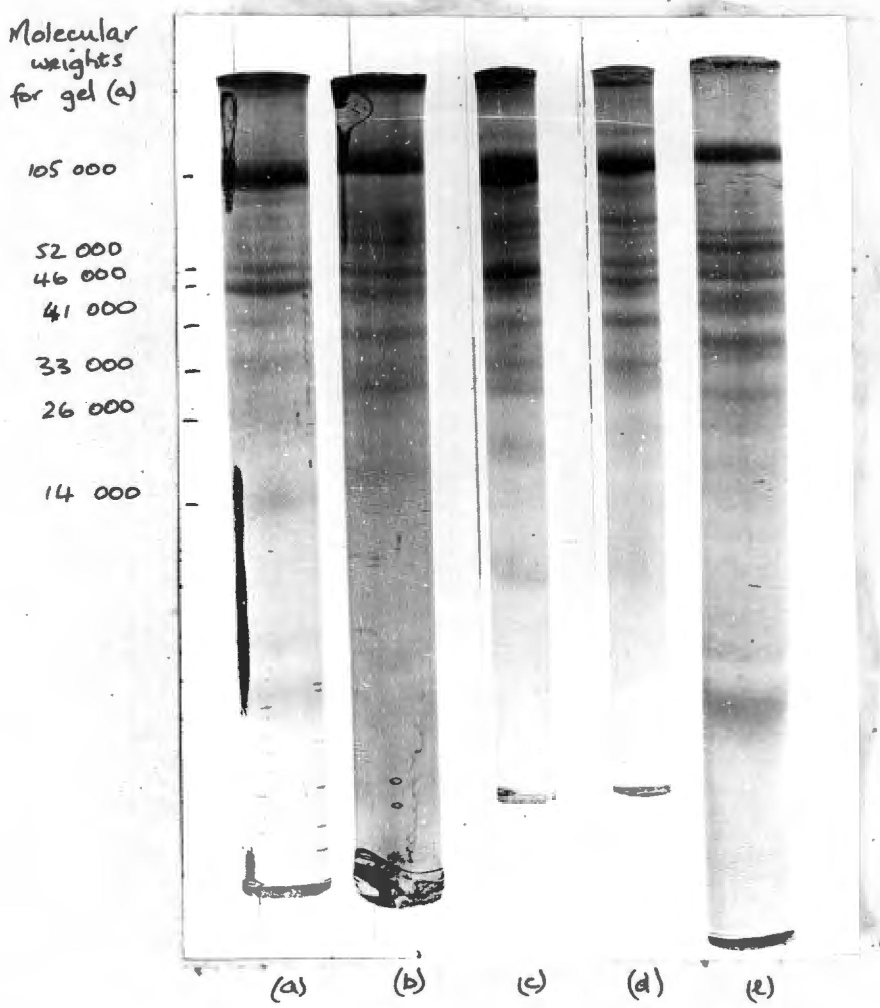


Fig. 21 Protein composition of fragmented sarcoplasmic reticulum from slowly-glycolysing muscle and from PSE muscle.

The FSR suspension used was harvested from continuous gradients (1,175-1,120 g/ml) and, in the case of PSE FSR, the material excluded the dense aggregated layer that invariably contaminated the more dense vesicles falling within these density limits (see fig. 15). FSR suspension (0,6 mg protein) was dissolved in 1% SDS and then the concentration of SDS was reduced to 0,1% by dialysis for 16 hrs. Approximately 0,02 mg protein was placed on the top of 10% polyacrylamide gels (containing inter alia 0,1% SDS) and electrophoresed so that a current of 8 ma per gel was maintained for approximately 2 hrs. The gel was extruded from the glass tubes and the protein bands fixed and stained in a solution containing 9,2% acetic acid, 50% methanol and Coomassie brilliant blue. (a) proteins of rabbit FSR, (b) and (c) of normal FSR and (d) and (e) of PSE FSR.

The molecular weights of the proteins were determined by a comparison of the relative mobilities of the bands with those of standards (see Fig.6).

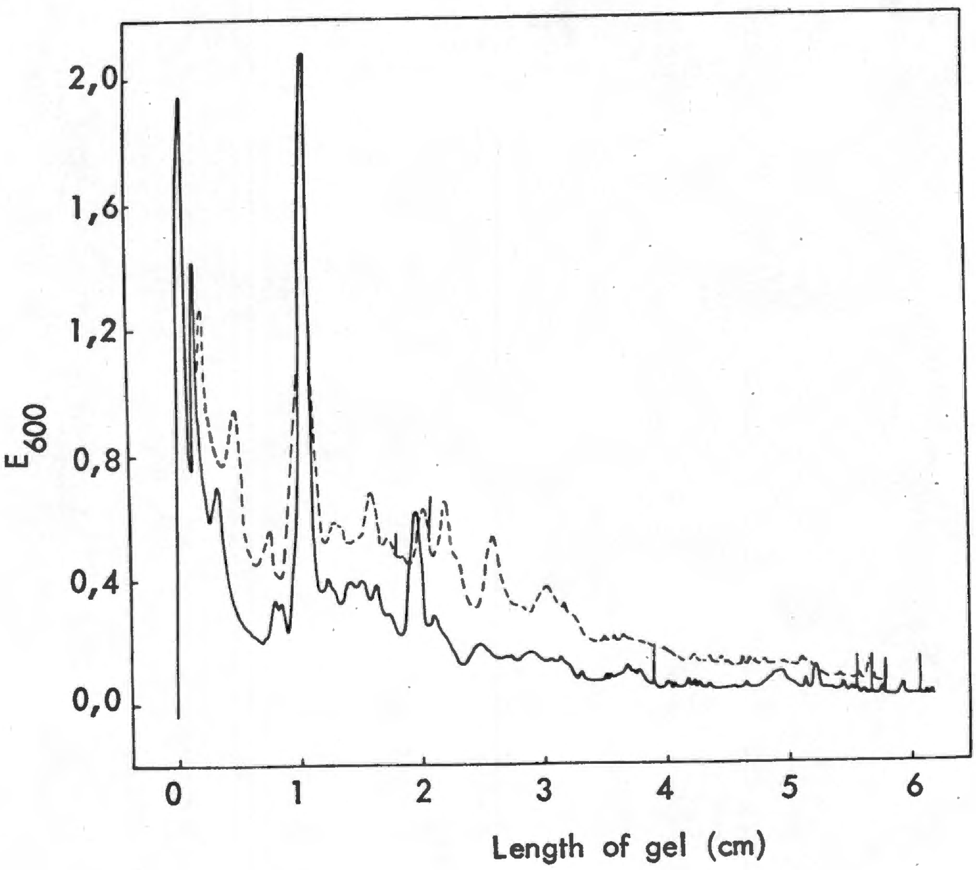


Fig. 22 Protein composition of fragmented sarcoplasmic reticulum: gel scan.

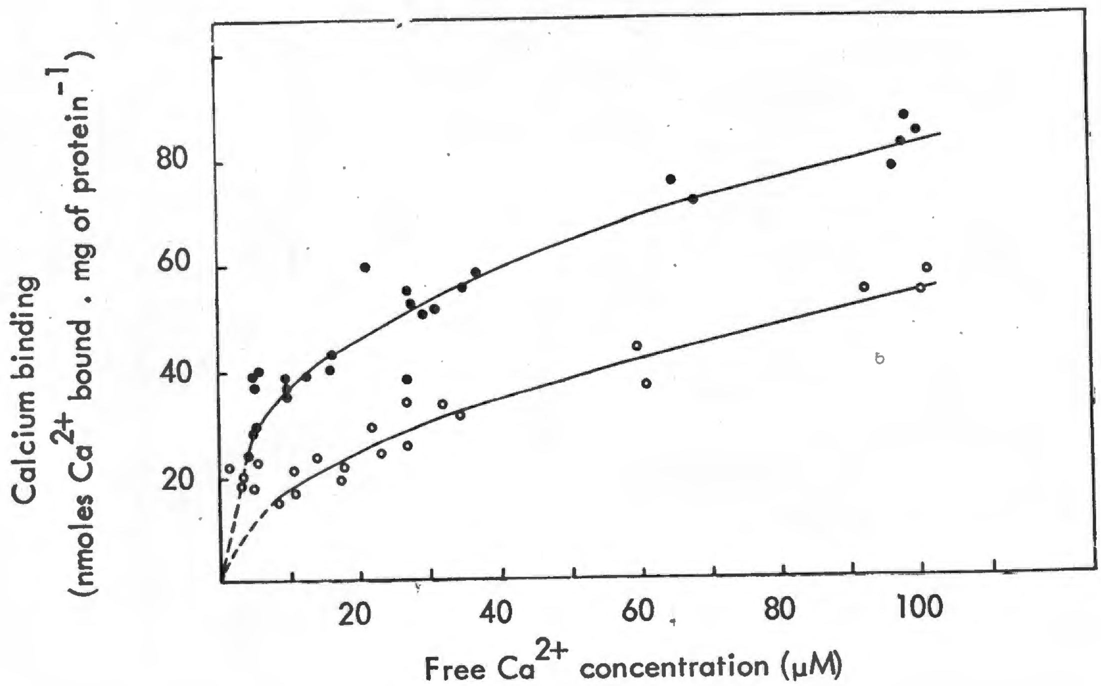
Absorption profile at 600 nm of stained gels (c) and (d) (see fig. 19) of FSR proteins from slowly-glycolysing (-) and from PSE (---) muscle.

rabbit skeletal muscle is included in fig 21 for comparison. The membrane proteins of FSR from porcine muscle appear to be similar to those described in the many studies on rabbit skeletal muscle. The only possible consistent difference lay in the relative ratio of the 55 000 component to calsequestrin. In rabbit muscle, calsequestrin is the more abundant component, being second only to the ATPase protein in amount. In porcine skeletal muscle FSR, the ratio of the 55 000 component to calsequestrin was equal to or greater than unity. The protein profile of membranes from FSR derived from rapidly-glycolysing muscle was similar to that from slowly-glycolysing muscle, in particular no breakdown of the ATPase band (into subunits of lower mol. wt.) was observed as has been described by Ostwald and MacLennan (1974) after tryptic digestion of the membranes.

3.8 PASSIVE CALCIUM BINDING TO AND CALCIUM EFFLUX FROM FRAGMENTED SARCOPLASMIC RETICULUM.

Summary: A binding site with high affinity for calcium (K_D=7,6 µM) and one of low affinity (K_D=120 µM) could be distinguished in the passive binding of calcium to FSR from slowly-glycolysing muscle. The capacities of these two sites were 68 nmoles Ca^{2+}. mg protein^{-1} and 202 nmoles Ca^{2+}. mg protein^{-1} respectively. FSR from PSE muscle possessed only one binding site, of intermediate affinity (K_D=31 µM) having the same capacity as the higher affinity site of control FSR. The rate constant of calcium efflux from preloaded vesicles in the presence of 6 mM EDTA was similar in FSR from the two muscle types. Efflux of calcium, from preloaded vesicles from both normal and PSE muscle proceeded more rapidly in the presence of ADP and Pi, than in their absence. The rate was greater from control vesicles.

Calcium binding to FSR vesicles in the absence of ATP and at low total cation concentration (1 mM KCl) was studied after equilibration and separation of the vesicles by high-speed centrifugation. FSR from slowly-glycolysing muscle binds large quantities of calcium ions (fig. 23). At 10 µM free calcium concentration approximately 40 nmoles Ca^{2+} was bound. Above this concentration, calcium appears to be bound by a relatively low-affinity process since at 100 µM free calcium concentration the amount of calcium bound (80 nmoles Ca^{2+} mg protein^{-1}) was only approximately double that at 10 µM. At all concentrations of free calcium, vesicles derived from PSE muscle bound approximately one-half as much as those from slowly-glycolysing muscle. The data for concentrations of calcium less than 7 µM were obtained



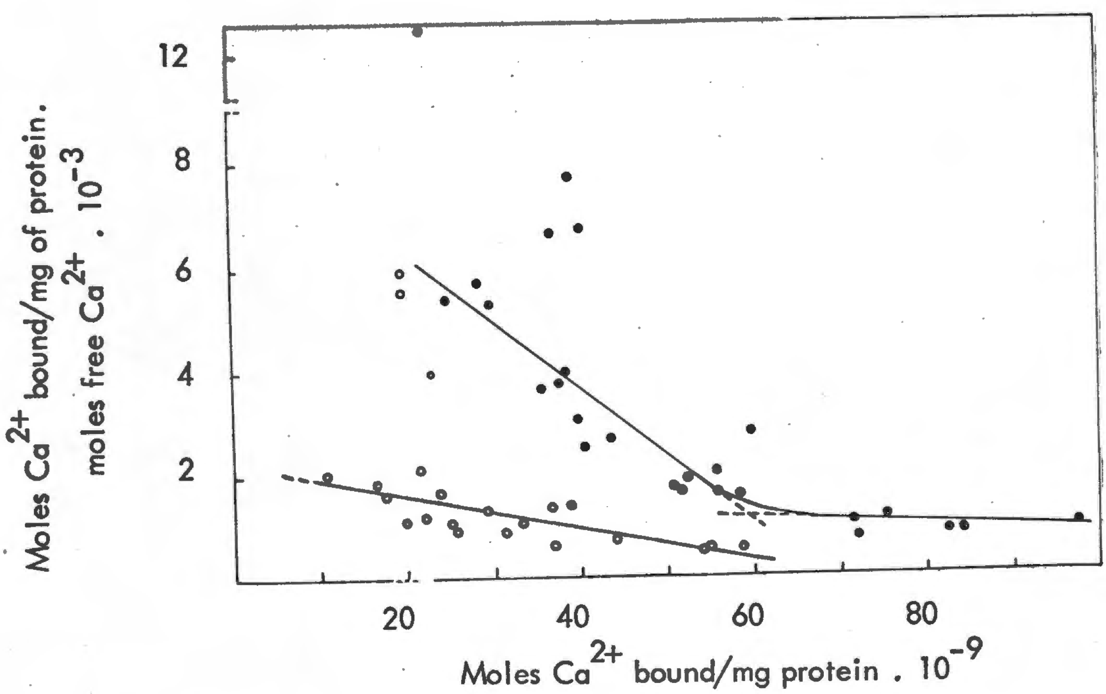


Fig. 23 Passive calcium binding to fragmented sarcoplasmic reticular membranes.

Aliquots of FSR protein suspension (0,20–0,25 mg protein) from N_2 and P_2 fractions (see fig. 10) were equilibrated with various Ca^{2+} concentrations for a minimum of 15 min and centrifuged at 106 500 xg for 10 min. The supernatant and the pellet (dispersed in 1 ml H_2O) were analysed for calcium by atomic absorption spectrophotometry in order to measure the free and bound calcium respectively. FSR from slowly-glycolysing muscle (●) and from PSE muscle (o). (a) Plot of amount of calcium bound per mg protein *versus* free Ca^{2+} concentration (b) Scatchard plot of data in (a).

in experiments in which no extra calcium was added to the suspension and the calcium measured was endogenous. Although it is presumed that during the equilibration period (15-60 min at 23°C), free exchange between bound and free calcium took place it is possible that a portion of bound endogenous calcium was nonexchangeable and this discrepancy could account for the deviation of the data points from the extrapolated curves to zero calcium concentration. Endogenous calcium has been shown by Carvalho (1966) to be about 20-30 nmoles Ca^{2+}. mg protein^{-1} for rabbit FSR. This fraction does not exchange readily with exogenous calcium. From a Scatchard plot (fig. 23b) of the data from fig 23a it appears that calcium binding to FSR from slowly-glycolysing muscle occurs at two binding sites. The higher-affinity binding site has a binding constant (K_D) of 7,6 μM and a capacity of 68 nmoles Ca^{2+}. mg protein^{-1}. The lower-affinity site has a K_D of 120 μM and a capacity of 202 nmoles Ca^{2+}. mg protein^{-1}. These two binding sites could correspond to the low affinity (K_D=13-32 μM) and nonspecific (K_D=320 μM) binding sites respectively described by Chevalier and Butow (1971) for FSR from rabbit skeletal muscle. These authors also described a third high-affinity site (K_D=0,4-1,3 μM) which could only be detected when endogenous calcium had been removed by a cation exchanger. The Scatchard analysis of the data from binding studies on FSR from PSE muscle revealed only one type of binding site (fig. 23) with an affinity (K_D=31 μM) intermediate between the medium- and low-affinity sites observed in slowly-glycolysing muscle. The total capacity (70 nmoles Ca^{2+}. mg protein^{-1}) was equivalent to that of the medium-affinity site of control muscle. All the data points obtained from fig. 23a have been included in the Scatchard plot from which it is obvious that the observations on samples which equilibrated in free concentrations of calcium $< 7\ \mu M$ were widely scattered possibly due to experimental error as a result of relatively large amounts of endogenously-bound calcium.

Passive Ca^{2+} efflux from Ca^{2+}-loaded FSR vesicles was studied following maximal accumulation of calcium into the vesicles in the presence of ATP and subsequent inhibition of the transport enzyme with 6 mM EDTA (fig. 24). Addition of EDTA resulted in the rapid loss of approximately 25% of accumulated calcium within 5 min. Subsequently, calcium efflux proceeded at a slow and in this particular experiment linear rate for at least a further 35 min. In a similar experiment using FSR from PSE muscle, maximum calcium accumulation was approximately one third of that from slowly-glycolysing muscle. Addition of EDTA also resulted in a rapid loss ($\pm$50%) of accumulated calcium. Subsequent efflux was slightly slower than that from control FSR. The first order rate constants calculated from the slopes of a semilogarithmic plot of the fraction remaining bound to membranes in the presence of EDTA versus time (fig.24b) were $1{,}1.10^{-2}\ min^{-1}$ and $1{,}0.10^{-2} min^{-1}$ for FSR from slowly-glycolysing muscle and from PSE muscle respectively. In other experiments, the rates were $5{,}3.10^{-2}$, $2{,}1.10^{-2}\ min^{-1}$ and $3{,}2.10^{-2}$, $2{,}3.10^{-2}\ min^{-1}$ respectively. The absolute and relative rates of efflux of calcium from FSR vesicles of PSE muscle origin do not appear to be increased.

The rate of calcium efflux was also studied in vesicles which had been loaded with calcium by equilibration against a medium containing a high free calcium concentration (20 mM). Efflux was then measured in the presence of ADP + Pi or in their absence (passive efflux). Over the time course of the experiment (40-50 sec), passive efflux in both normal and in PSE vesicles was

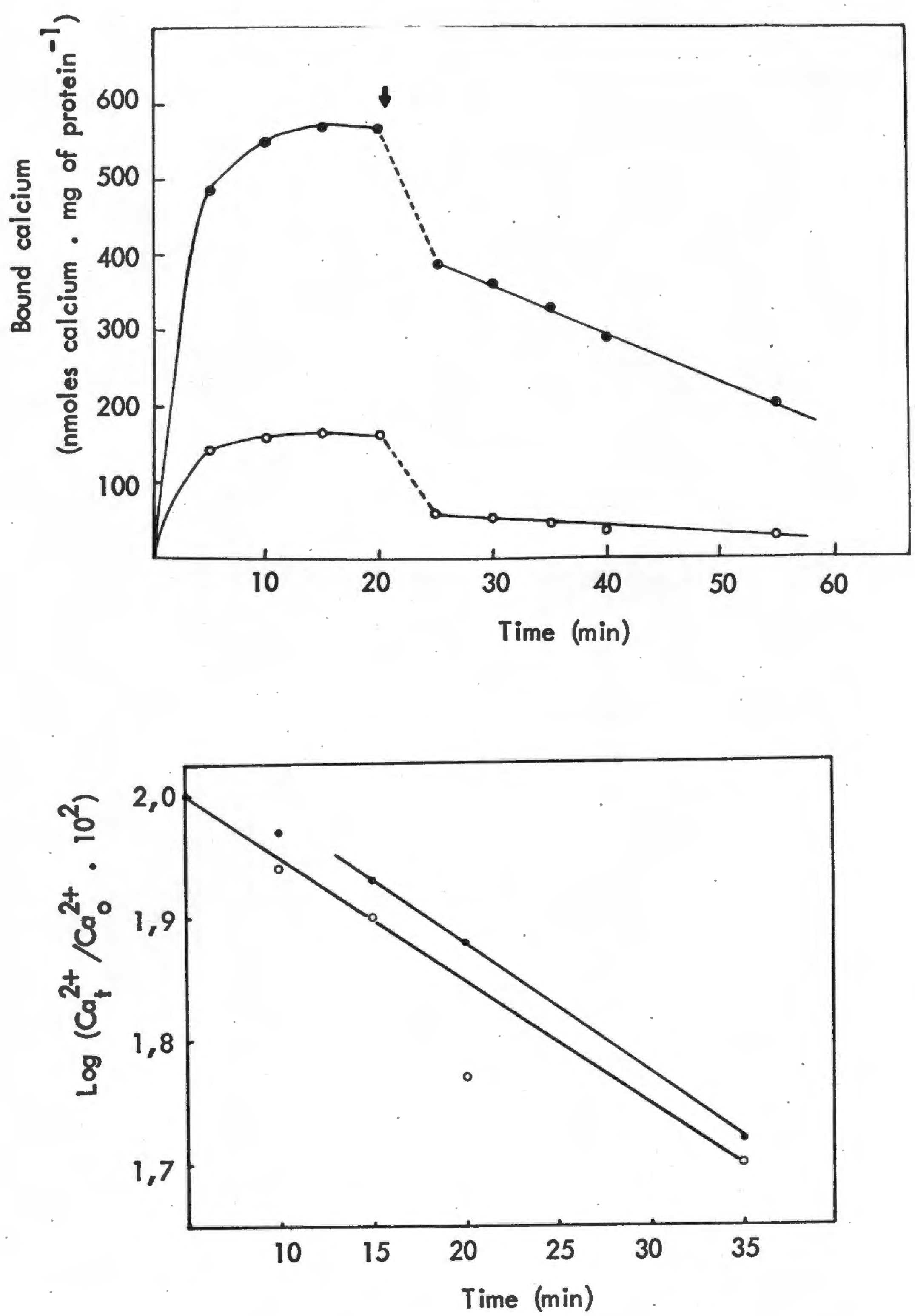


Fig. 24 Passive calcium efflux from fragmented sarcoplasmic reticulum preloaded with calcium in the presence of ATP.

FSR vesicles (0,68 and 0,72 mg protein) were loaded with calcium in a medium containing 50 mM KCl, 20 mM histidine, pH 7,2, 5 mM $MgCl_2$, 0,5 mM$^{45}CaCl_2$, 0,5 mM EGTA, 5 mM ATP and 5 mM K-oxalate. Efflux was initiated at 20 min by the addition of EDTA (final conc. 6 mM). The logarithmic plot represents log of the fraction of calcium lost at time t ($Ca^{2+}t$) after addition of EDTA (Ca^{2+}_o) versus time. (•), vesicles from slowly-glycolysing muscle (N_2) and (o), vesicles from PSE muscle (P_2).

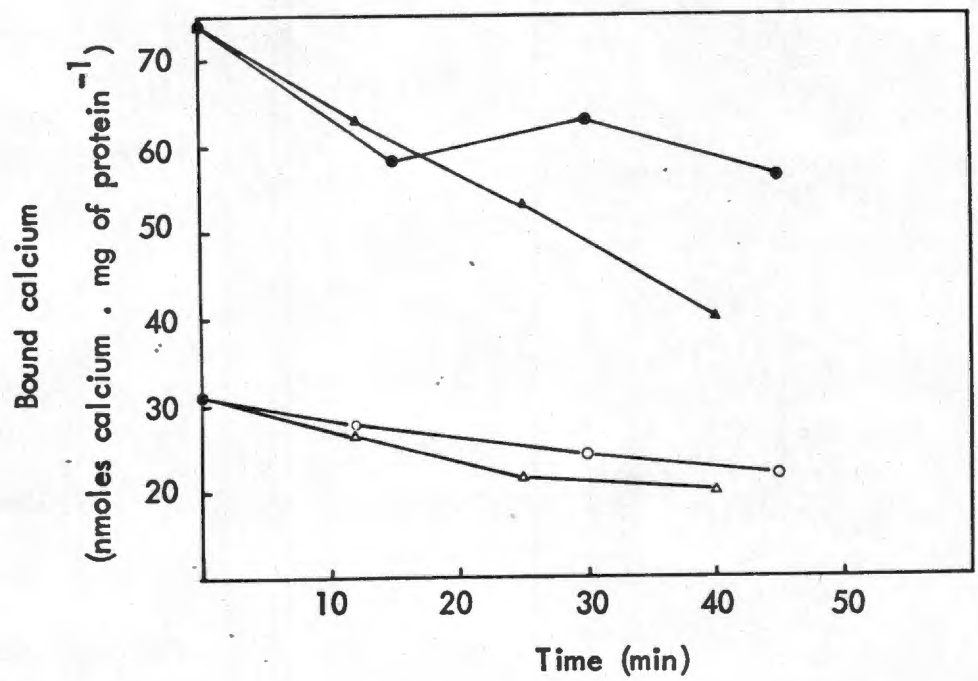


Fig. 25 Calcium efflux from fragmented sarcoplasmic reticular vesicles preequilibrated with high concentrations of calcium.

Protein suspensions (6-8 mg) were preincubated overnight with 0,1M KCl, 5 mM histidine, pH 7,2, 5 mM $MgCl_2$ and 20 mM $^{45}CaCl_2$ at 3-4°C. Efflux was initiated by a 20 fold dilution of the "loaded" FSR with a diluting medium equilibrated at 25°C. Diluting media consisted of 0,1M KCl, 5 mM histidine, pH 7,0, 5 mM $MgCl_2$, 10 mM EGTA (circles) and as before but including 1,0 mM ADP and 3,0 mM Pi (triangles). Efflux was followed by filtering 0,2 ml aliquots at indicated times and determining the residual radioactivity remaining on the filter paper after washing with 0,1M KCl, 5 mM histidine, pH 7,0, buffer. The value for zero time was obtained by measuring efflux after 12 sec at 0°C in a diluting medium that did not contain ADP and Pi. (•, ▲) FSR from slowly-glycolysing muscle (N_2) and (o, △) FSR from PSE muscle (P_2).

slow. In the presence of ADP + Pi efflux was accelerated from normal FSR. The PSE vesicles bound less calcium (approx. $^{1}/3$ of control values) and efflux was only slightly stimulated in the presence of ADP and Pi.

These experiments were done in the presence of 0,1M KCl which could effectively compete with calcium for the low affinity passive binding site found in control FSR (fig. 23) and therefore the binding of calcium is to calcium-specific sites (Chevalier and Butow, 1971). Zero time shows a capacity for control FSR of approximately 70-75 nmoles Ca^{2+} bound. mg $protein^{-1}$. This value agrees with that obtained for the capacity of the medium-affinity binding site (70 nmoles Ca^{2+}. mg $protein^{-1}$) calculated from the Scatchard plot of passive calcium-binding (fig. 23). The total calcium capacity obtained from PSE FSR in the dilution experiments (approx. 30 nmoles Ca^{2+} bound. mg $protein^{-1}$) does not agree with the capacity of the single binding site found from the Scatchard analysis (approx. 70 nmoles Ca^{2+} bound. mg $protein^{-1}$). Possibly the Scatchard data for PSE may therefore represent more than one binding site, with both calcium-specific and calcium-nonspecific varieties.

3.9 THE TEMPERATURE-DEPENDENCE OF CALCIUM ACCUMULATION AND OF ATPASE ACTIVITY OF FRAGMENTED SARCOPLASMIC RETICULUM ISOLATED FROM SLOWLY-GLYCOLYSING AND FROM PSE MUSCLE.

Summary: An apparent activation energy of 10,5 kcal/mole was found for the Ca-Binding process of FSR from slowly-glycolysing muscle. Due to the large scatter of data for this process in FSR from PSE muscle, it was not possible to derive a meaningful ΔH act. value. The Ca-Uptake process of FSR from both slowly-glycolysing and from PSE muscle displayed an activation energy approximately double that for Ca-Binding. The kinetics of both processes appeared to be similar for FSR from both muscle types, except that control FSR exhibited an increased calcium efflux rate above 35°C from preloaded vesicles than those of PSE origin. An Arrhenius plot of ATPase activity of fractions N_2 and N_3 from slowly-glycolysing muscle revealed discontinuities at approximately 17°C and 32°C and processes having activation energies of approximately 10,6 and 26,0 kcal/mole were apparent. Fractions N_1 from control muscle and P_2 and P_3 from PSE muscle did not exhibit a discontinuity in the higher temperature range and the change in ATPase activity from 20°C to 45°C could be described by a single process with an activation energy of approximately 14,5 kcal/mole. Fragments N_1 and N_2 contained an ATPase which underwent rapid isothermal inactivation at 51,5°C (rate constant approx. 0,17 min^{-1}) whereas N_3, P_1 and P_3 were more resistent to thermal inactivation (rate constant approx. 0,07 min^{-1}).

The purified fractions of FSR isolated by bouyant-density centrifugation on continuous sucrose gradients and having the highest ATP-dependent calcium accumulation and Ca^{2+}-dependent ATPase activity (ie N_2 and P_2, $1,158 < \rho < 1,184$ g/ml) were used to follow the temperature dependence of calcium accumulation over the temperature range 0-40°C. Both Ca-Binding, in the absence of oxalate, and Ca-Uptake in the presence of 5 mM oxalate were determined (fig 26).

The initial rapid rate of accumulation (0-15 sec) was markedly dependent on temperature between 0°C and 20°C. Above 20°C binding of calcium at 15 sec remained constant over the range 20-40°C. After 1 min, Ca-Binding proceeded at a slow but similar rate in the range 0°C to 30°C. At 35°C and 40°C the amount of calcium bound at 5 min declined to a small but significant extent. The Ca-Binding activity of PSE membranes was approximately $^1/_{20}$ of control preparations. On account of this low activity there was considerable scatter in the data but it appears that the kinetics of Ca-Binding is similar to that of control preparations. Binding was maximal at 15°C and declines to a constant level between 20°C and 40°C. The Arrhenius plot of "initial" rates of Ca-Binding (measured at 15 sec) was a straight line with ΔH=10,5 kcal/mole. There is considerable scatter of the points in the Arrhenius plot for PSE Ca-Binding since Ca-Binding in the range 0-40°C does not progressively increase with rising temperature.

The rate of Ca-Uptake (in the presence of oxalate) was virtually nil at 0°C but became influenced maximally at higher temperatures, up to 40°C. At 15°C and 20°C calcium was still being taken up after 10 min. Between 30°C and 40°C maximal accumulation was reached at 3-5 min and declined thereafter particularly at 40°C where approximately half the accumulated calcium had been lost again

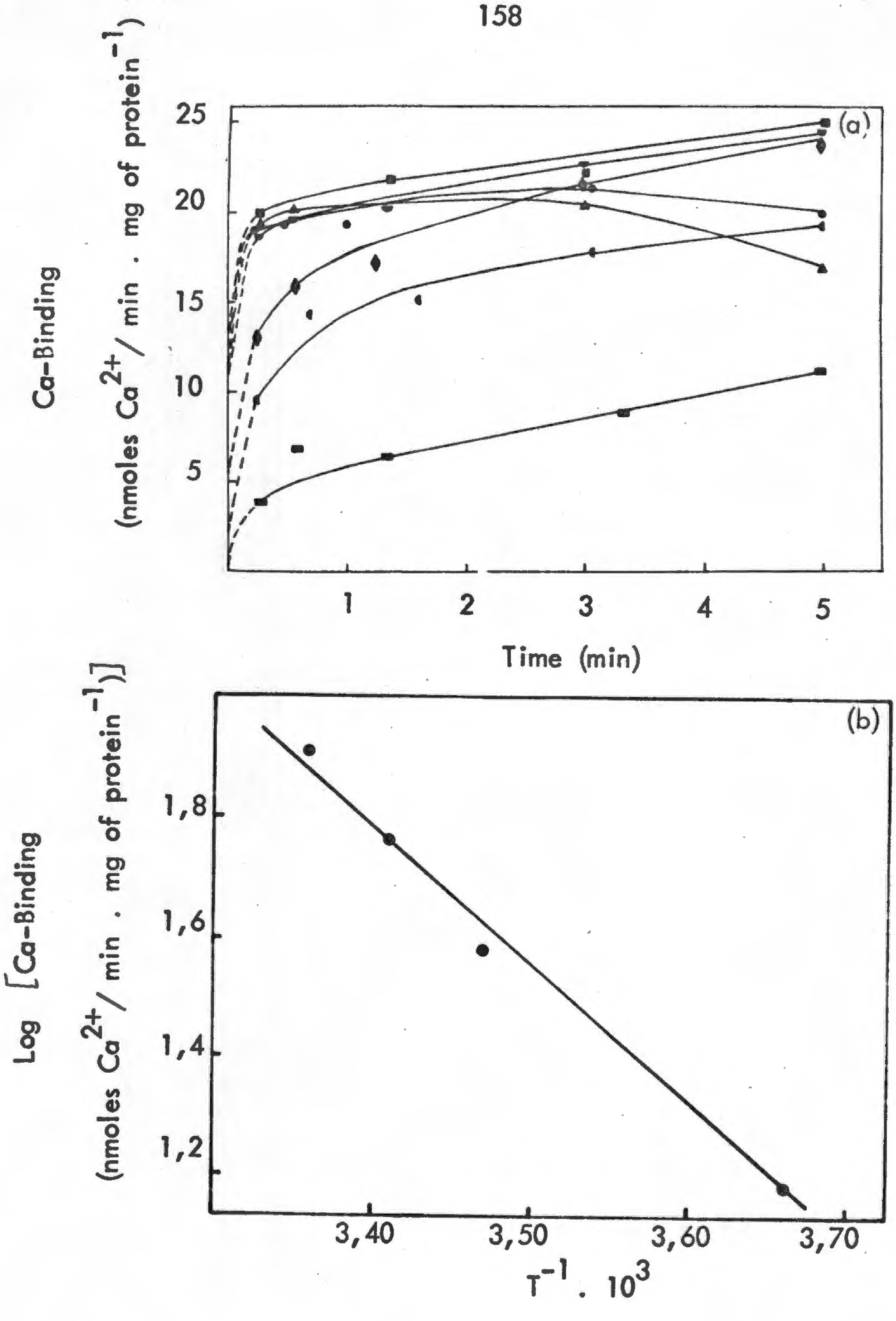
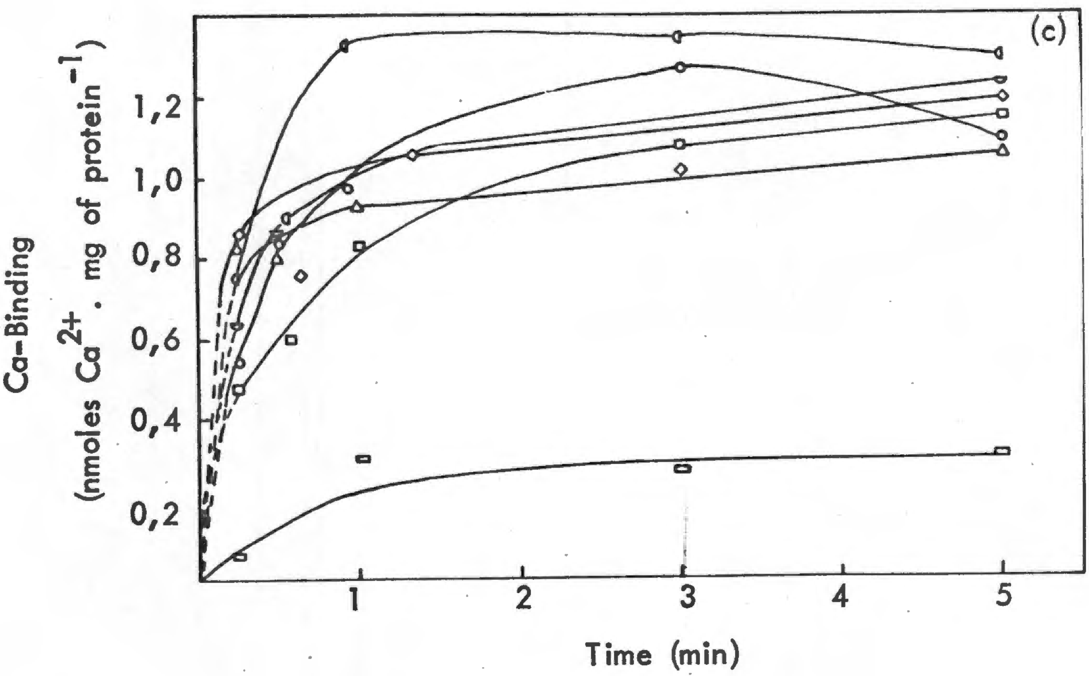


Fig. 26 Temperature-dependence of Ca-Binding and Ca-Uptake of calcium by fragmented sarcoplasmic reticulum.

Ca-Binding and Ca-Uptake were measured at various temperatures on fractions N_2 and P_2 as described in Table 4. (a) Ca-Binding by FSR from slowly-glycolysing muscle (▬), 0°C; (◖), 15°C; (◆), 20°C: (■), 25°C; (●), 30°C; (▲), 35°C: (▾), 40°C). (b) Arrhenius plot of Ca-Binding by FSR from slowly-glycolysing muscle (data obtained from Ca-Binding at 15 sec).



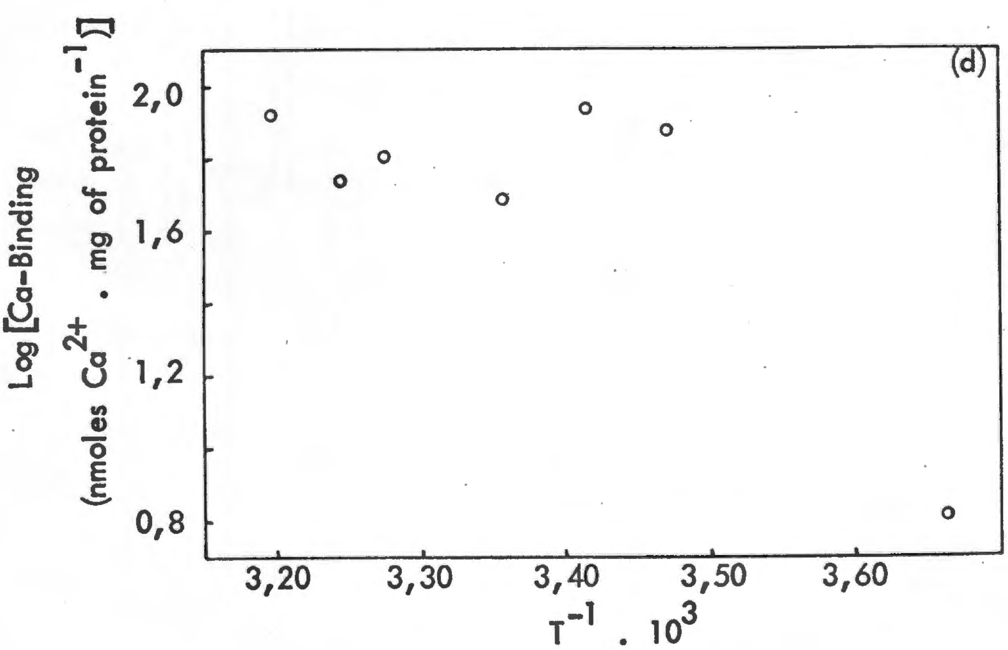
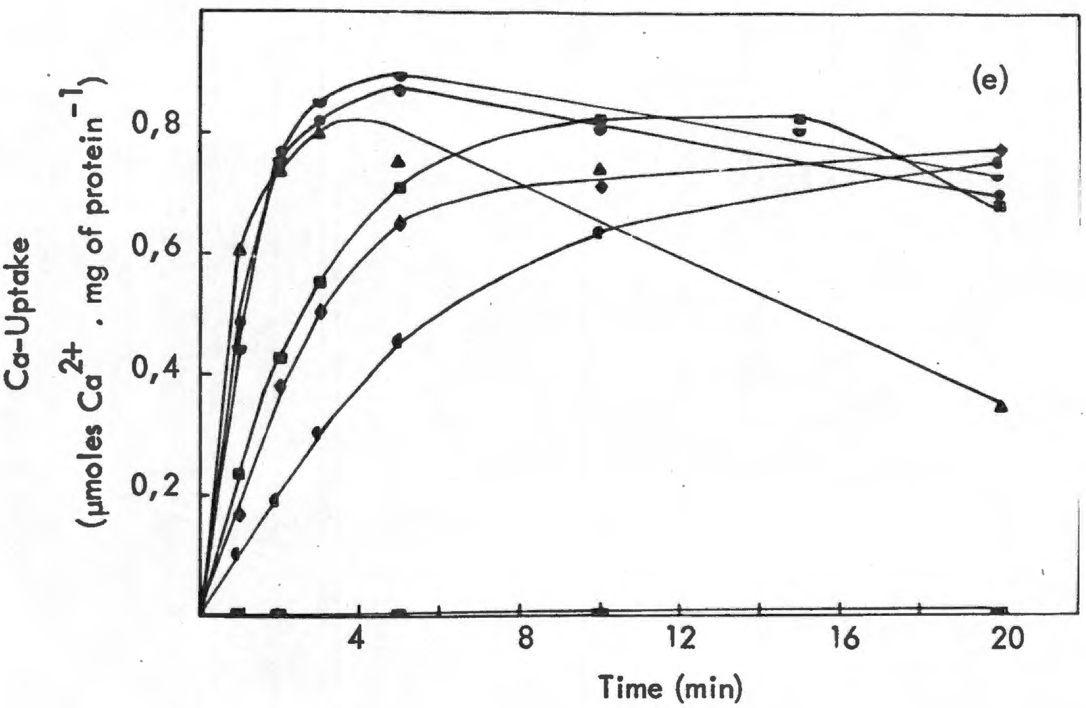


Fig. 26 cont. (c) Ca-Binding by FSR (0,27 mg protein) from PSE muscle (▭ , 0°C; ◖ , 15°C; ◊ , 20°C; □ , 25°C; o , 30°C; ▵ , 35°C; ◡ , 40°C) (d) Arrhenius plot of Ca-Binding by FSR from PSE muscle (data obtained from Ca-Binding at 15 secs).



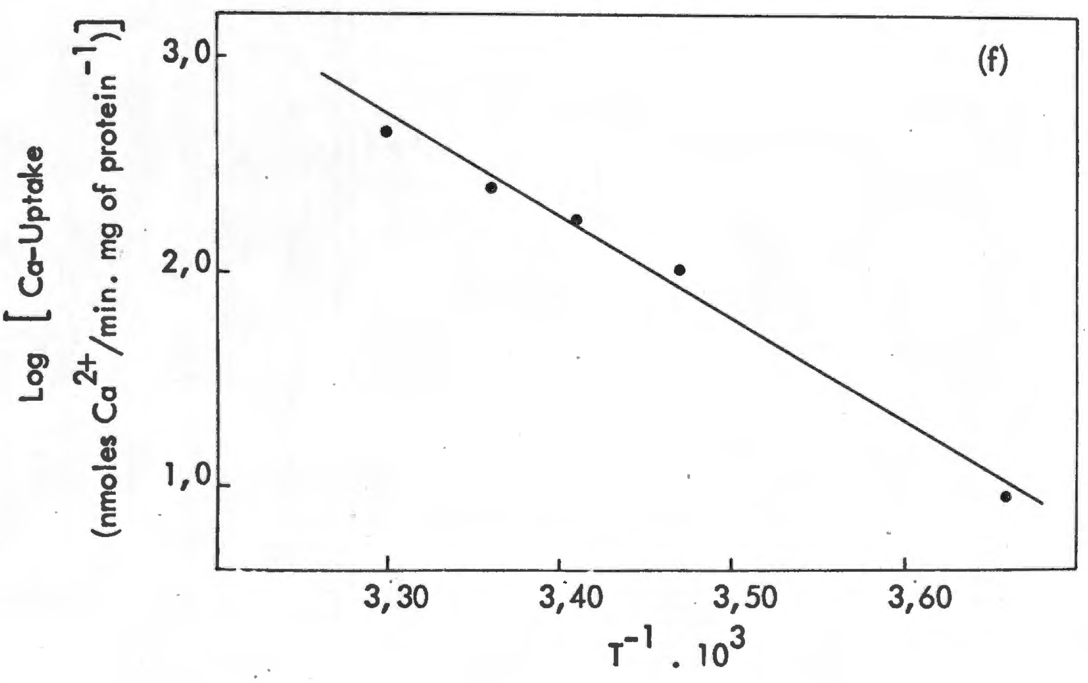


Fig. 26 cont. (e) Ca-Uptake by FSR (0,22 mg protein) isolated from slowly-glycolysing muscle. (f) Arrhenius plot of initial rates of Ca-Uptake by FSR from slowly-glycolysing muscle.

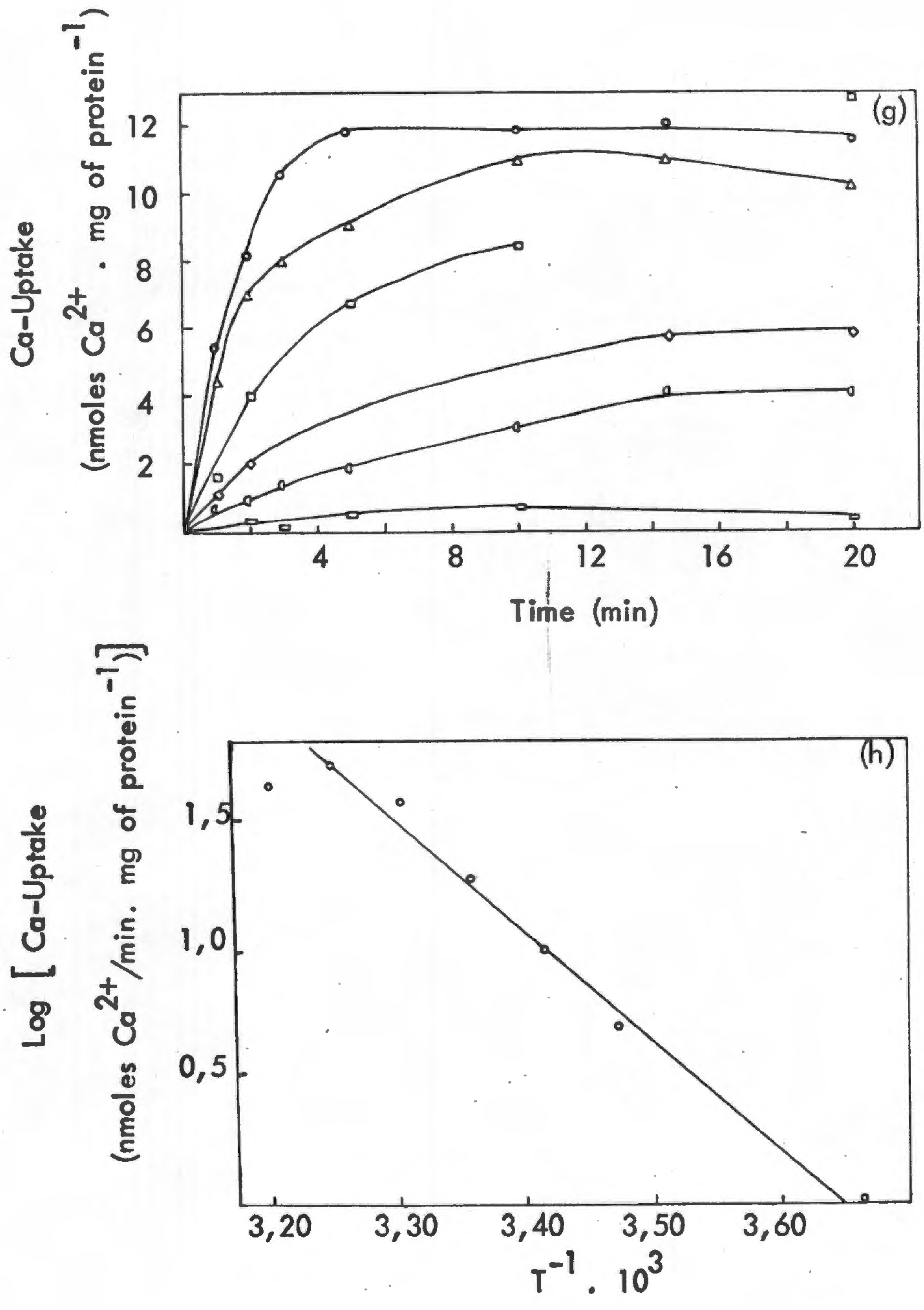


Fig. 26 cont. (g) Ca-Uptake by FSR (0,29 mg protein) isolated from PSE muscle. (h) Arrhenius plot of initial rates of Ca-Uptake by FSR from PSE muscle.

after 20 min. The activation energy derived from an Arrhenius plot (fig 26) was 22,0 kcal/mole. No discontinuity could be discerned in the plot. Inesi _et al_. (1973) have recognised a discontinuity at $\pm 19^{\circ}C$ with FSR from rabbit muscle with a lower activation energy above this point (17 kcal/mole). The best fit of a line through the points at 15, 20, 25 and $30^{\circ}C$ gives a value for ΔH of 16,4 kcal/mole which is comparable to that found by Inesi _et al_.

Ca-Uptake by PSE FSR was approximately 50-100 times lower than that of the control. This difference is more pronounced than was usually found (5 to 10 times less). Maximal accumulation occurred after 20 min of reaction time. Efflux at $40^{\circ}C$ was minimal after 20 min. Arrhenius plot of Ca-Uptake by PSE membranes gave a comparable activation energy to the control (ΔH=19,6 kcal/mole).

The effect of temperature on Ca^{2+}, Mg^{2+}-ATPase activity of preparations of purified FSR was assayed by the pH stat method at pH 7,0. The temperature was raised continuously from $10^{\circ}C$ to $55^{\circ}C$ by means of a waterbath in an approximately linear manner with time. A temperature-compensating device which incorporated a thermistor probe was inserted in series with the calomel electrode to compensate for drift of the glass electrode with temperature and adjusted so that, in the absence of enzymatic activity, apparent addition of H^{+} ions equivalent to 0,01 µmoles Pi liberated min^{-1} was generated. The results of a typical temperature-programmed experiment are shown in fig 27. ATPase activity was calculated from tangents to the continuous record of volume of titrant (NaOH) added.

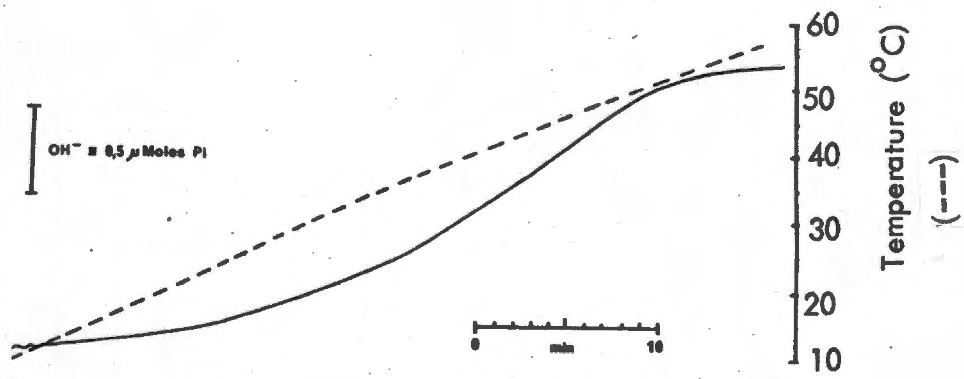


Fig.27 NaOH-titration curve obtained using pH stat method to measure ATPase activity during continuous temperature programming.

The pH-stat titration method was employed with temperature programming to determine the effect of temperature on Ca^{2+}-dependent ATPase activity of FSR. The assay medium consisted of 0,1M KCl, 5 mM $MgCl_2$, 0,1 mM EGTA, 0,1 mM $CaCl_2$, 10 mM ATP in a total volume of 3,0 ml. The reaction mixture equilibrated at 10°C in an atmosphere of CO_2-free N_2. The reaction was initiated by adding 0,25 mg FSR protein and temperature rise initiated by disconnecting the waterbath thermostat. The amount of NaOH added (14,5 mM) was recorded against time. Temperature was monitored by means of a thermistor probe.

Three fractions from slowly-glycolysing muscle N_1, N_2, N_3 and the two fractions from PSE muscle P_2, P_3 were analysed by this method (fig 28). Arrhenius plots of the data are presented in figs. 28b,d and f.

Hydrolysis rate of particles of greatest bouyant-density, N_1, increased logarithmically in the temperature range 10°C to 38°C, with an apparant single activation energy of 16,5 kcals/mole (fig 28a) A rise in temperature from 40 to 46°C did not alter the high hydrolysis rate achieved at the former temperature. Temperatures above 46°C resulted in a decline in ATPase activity due to rapid inactivation of the enzyme. ATPase activity of fraction N_2 was more sensitive to temperature than N_1 in the temperature range 40-50°C. The ATPase activity in the corresponding fraction from PSE muscle, P_2, was higher in the lower temperature ranges ($<$40°C) and lower above this temperature than N_2, with a cross-over point at 39,7°C. The activity of PSE membranes appears to be less sensitive to inactivation at high temperatures (see below). Similar curves were observed for N_3 and P_3, for normal and PSE membranes respectively, except that the maximal activities of these fractions were similar.

Arrhenius plots of the data for various fractions are presented in fig 28 and a summary of activation energies are given in table 14.

Single activation energies can describe the temperature-dependence of ATPase activities in N_1 and P_2. Both N_2 and N_3 have discontinuities at approximately 32°C and 17°C which demarcate a low-activation energy process between these temperatures from two processes of higher activation energy above and below the inflection points. The higher activation energy process above 32°C is absent from both P_2 and P_3. The results of Inesi et al. (1973) with Ca^{2+}, Mg^{2+}-dependent ATPase of purified rabbit skeletal muscle

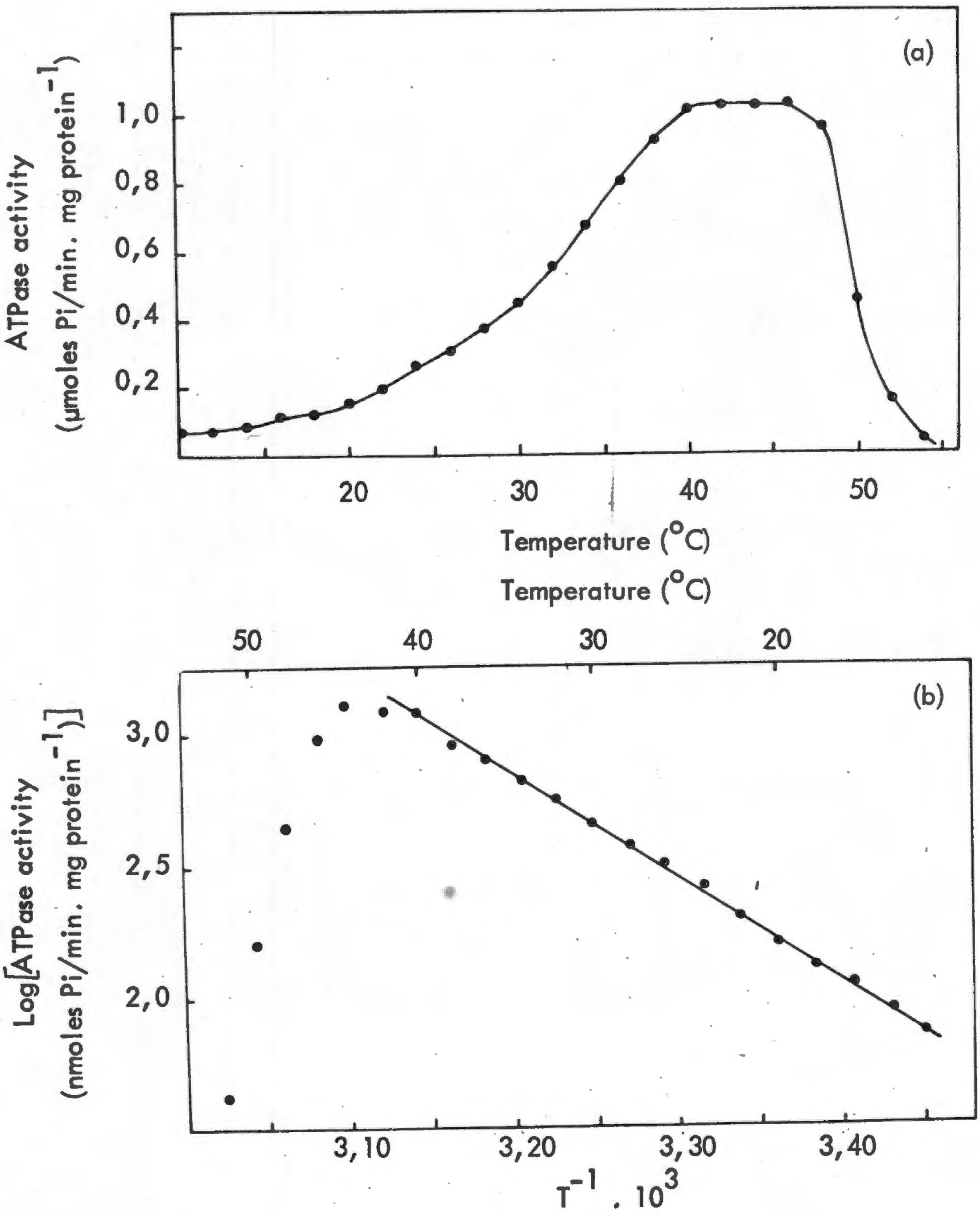


Fig. 28 Temperature-dependence of ATPase activity of fragmented sarcoplasmic reticulum.

ATPase activity of FSR fractions harvested from a discontinuous sucrose gradient (see fig. 10) was measured by means of pH stat method with continuous temperature programming as described in fig. 27 . ATPase activity of FSR from slowly-glycolysing muscle (●) and from PSE muscle (o). (a) Fraction N_1, (b) Arrhenius plot of data shown in (a).

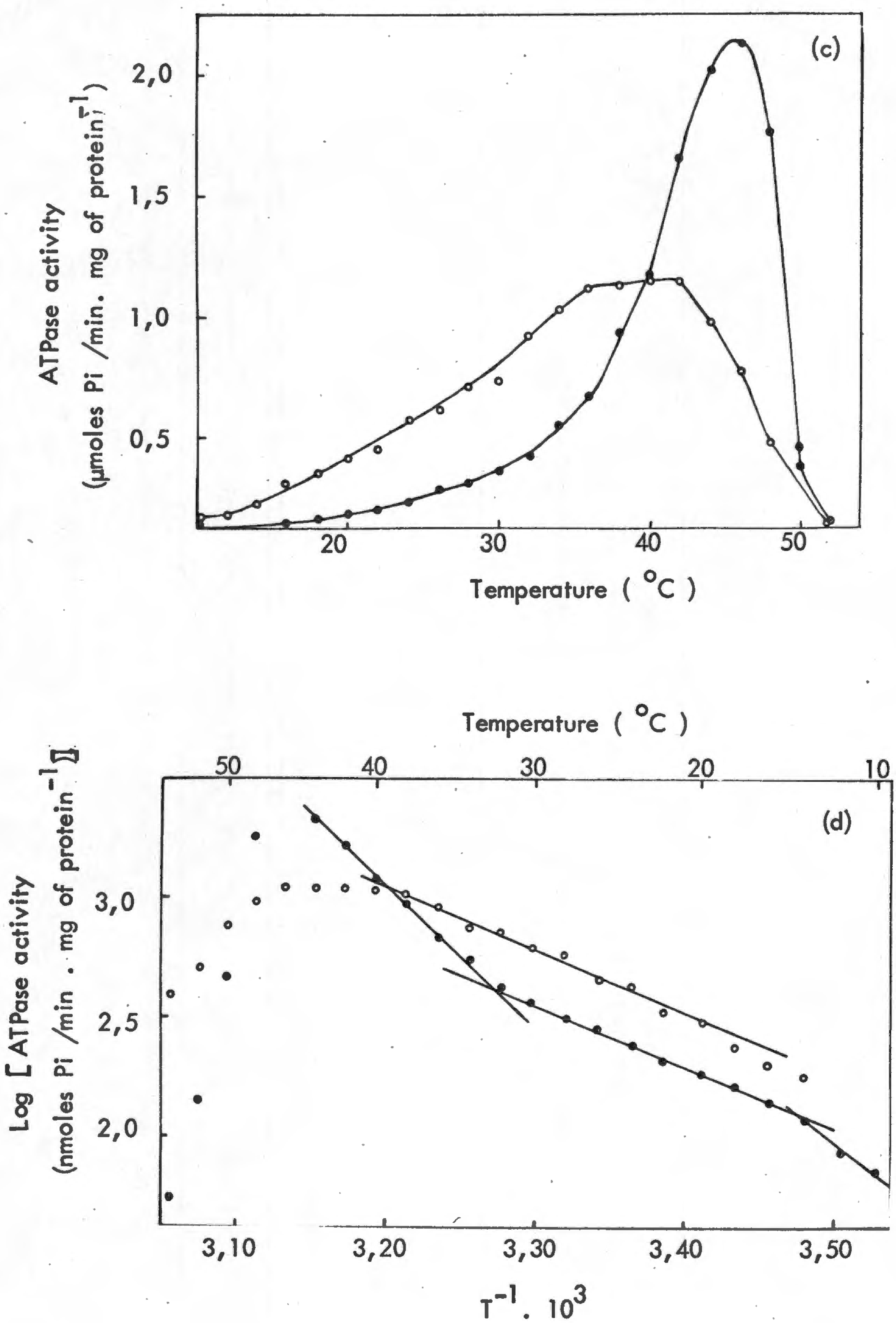


Fig. 28 cont. (c) Fractions N_2 and P_2, (d) Arrhenius plot of data shown in (c). FSR from slowly-glycolysing muscle (•) and from PSE muscle (o).

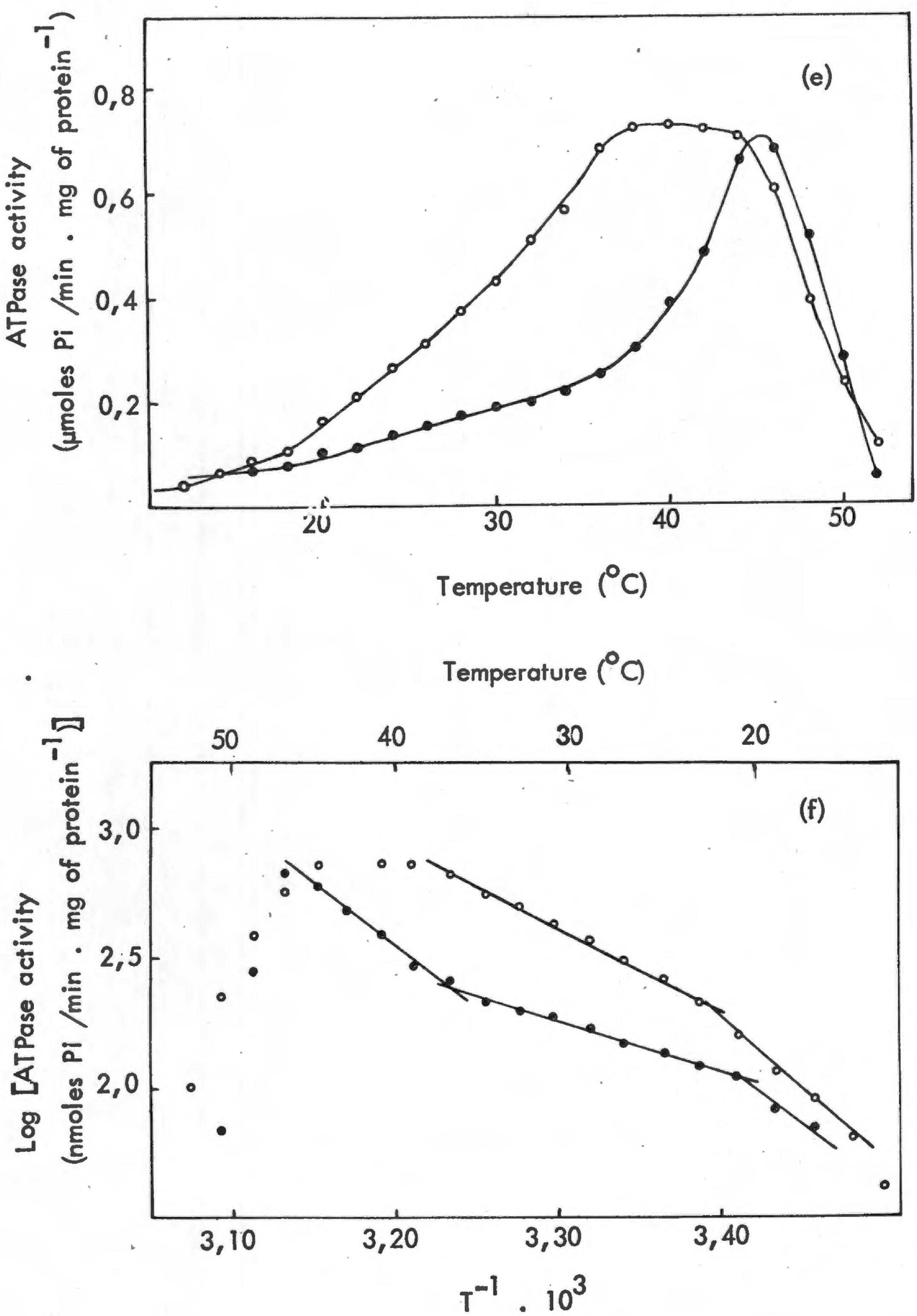


Fig. 28 cont. (e) Fractions N_3 and P_3. (f) Arrhenius plot of data shown in (e). FSR from slowly-glycolysing muscle (•) and from PSE muscle (o).

Table 14 Activation energies of the process of calcium accumulation and of ATPase activity in purified fractions of fragmented sarcoplasmic reticulum.

The data given summarise the results of figs. 26 and 28.

	ΔH (k cal/mole)				
	N_1	N_2	P_2	N_3	P_3
Ca-Binding	-	10,5	-	-	-
Ca-Uptake	-	22,0	19,6	-	-
Ca^{2+}, Mg^{2+}-ATPase activity	16,5	29,9/12,3	12,3	22,1/8,8	14,8/24,9

FSR are similar to those observed here for the fraction N_2 which has been observed to have the highest specific activity. The high activation energy process below 21°C in P_3 corresponds to that seen in N_2 and N_3.

Therefore, the major alteration in temperature-dependence of ATPase activity in FSR from rapidly-glycolysing muscle is the loss of the process of high activation energy between 32°C and 45°C which is evident in FSR membranes prepared from slowly-glycolysing muscle.

The susceptibility of Ca^{2+}, Mg^{2+}-dependent ATPase to isothermal heat-inactivation has been studied by means of the pH stat method in which the time course of ATP hydrolysis was followed at 51,5°C and pH 7,0 over 20 min. During this period no more than 20% of the total ATP in the assay medium was hydrolysed.

If the inactivation is assumed to be first order with respect to ATPase activity then the rate of inactivation of ATPase activity is given by

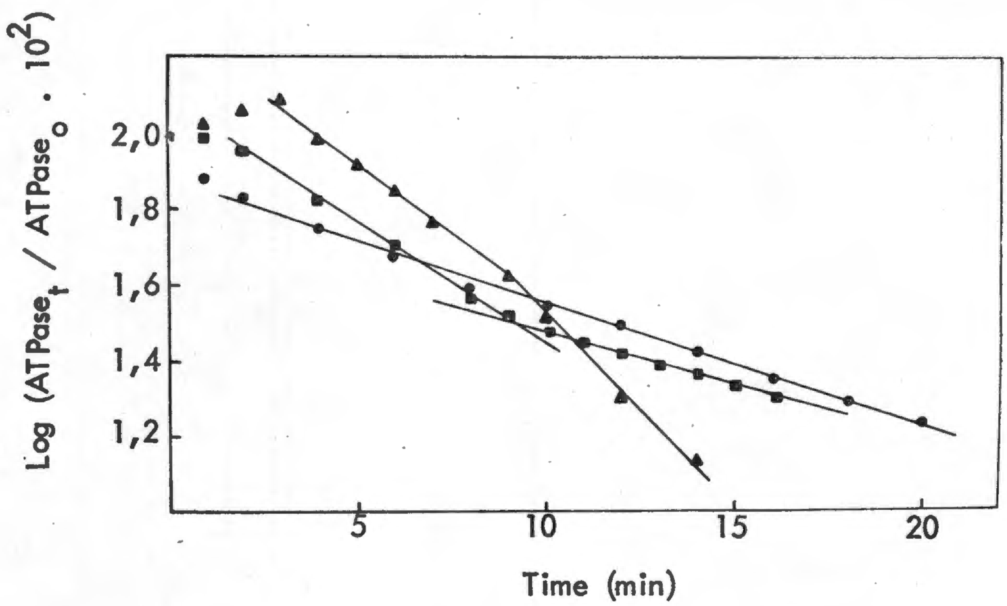
$$\frac{dA}{dt} = kA$$

Where A is the concentration of active ATPase molecules (proportional to the activity) and k is the rate constant of inactivation. By integrating

$$At = Ao\,e^{-kt}$$

or

$$\log \frac{At}{Ao} = \frac{-kt}{2,303}$$



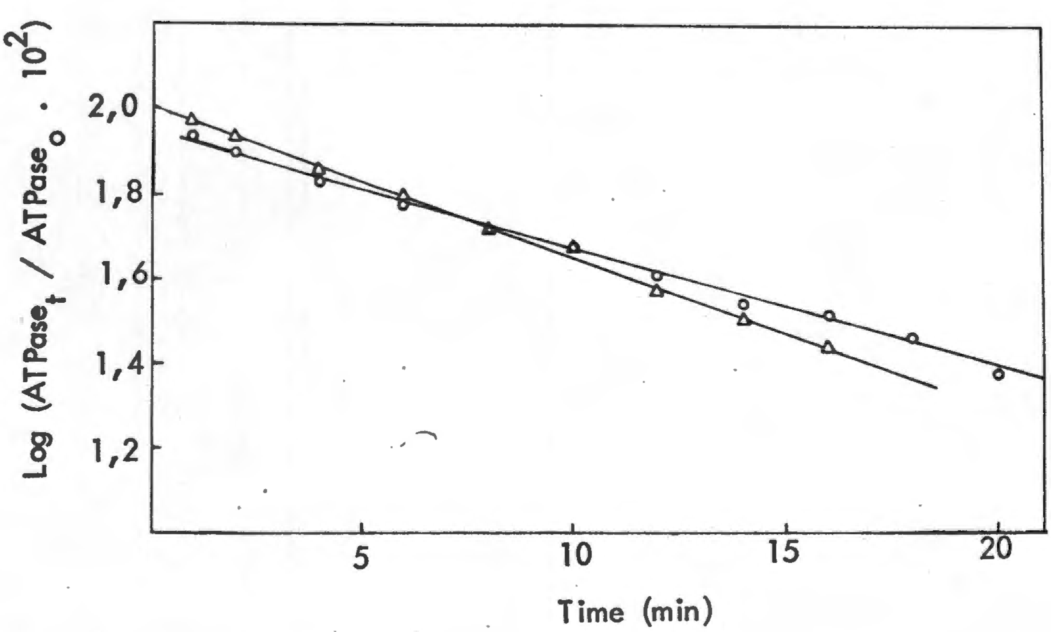


Fig. 29 Isothermal inactivation of Mg^{2+}, Ca^{2+}-dependent ATPase activity of fragmented sarcoplasmic reticulum.

ATPase activity of FSR (0,01–0,16 mg protein) in fractions N_1 (■), N_2 (▲) and N_3 (●) from slowly-glycolysing muscle and P_2 (△) and P_3 (○) from PSE muscle was measured at 51,5°C by the pH stat method and was expressed as µmoles Pi liberated/min. mg of protein^{-1} at zero time (ATPase$_o$) and at varying times thereafter (ATPase$_t$).

Table 15 Rate constants and half-life values of thermal inactivation process of Ca^{2+}, Mg^{2+}-ATPase activity of fragmented sarcoplasmic reticulum.

The values were calculated from slopes of the curves in fig. 29.

Fraction	Rate constant (min^{-1})		$t\frac{1}{2}$ (min)	
	first process	second process	first process	second process
N_3	0,072	-	9,6	-
P_3	0,080	-	8,7	-
N_2	0,18	0,23	3,9	3,0
P_2	0,066	-	10,5	-
N_1	0,15	0,067	4,6	10,3

Where Ao is the activity at time t=o and At the activity after time t. The half-life period for the inactivation process during which the activity has decreased to the half of its original activity is that time when

$$\frac{At}{Ao} = 0,5$$

$$\text{ie } t_{\frac{1}{2}} = \frac{(-\log 0,5)(2,303)}{k}$$

$$= \frac{0,693}{k}$$

A relatively rapid phase of inactivation in fractions N_1 and N_2 was followed in the former by a slower decline and in the latter by a slightly enhanced rate. A single, slower, first-order rate constant adequately describes the inactivation process in N_3. The inactivation process in both fractions P_2 and P_3 can be described by a single, first-order process with rate constants of 0,060 and 0,080 min^{-1} respectively, which correspond to the slower rate constant observed in N_3 (0,072 min^{-1}) and the latter phase of inactivation in N_1 (0,067 min^{-1}).

It is apparent, therefore, that most of the ATPase activity in N_2 and a considerable fraction in N_1 has a high susceptibility to thermal inactivation. All the ATPase activity present in fractions P_2 and P_3 derived from PSE muscle is relatively resistent to thermal inactivation.

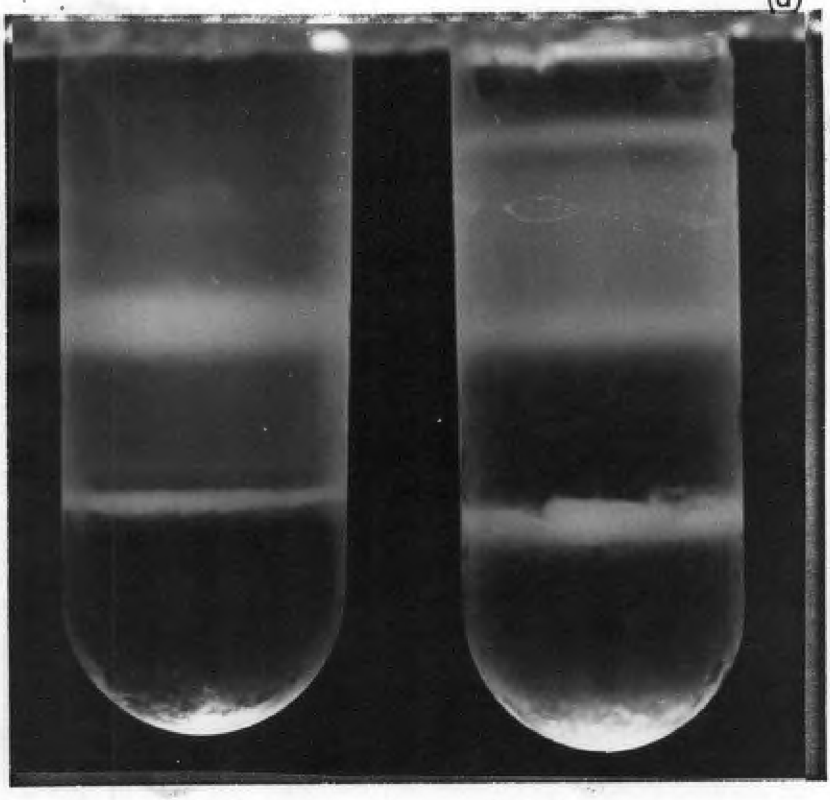
TREATMENT WITH ACID.

Summary: Incubation of a homogenate of slowly-glycolysing muscle at pH 5,5 for 10 min at 37^0C resulted in a marked loss in calcium accumulating ability of isolated FSR. The associated Mg^{2+}, Ca^{2+}-dependent ATPase activity on the other hand was slightly enhanced by this treatment. The banding pattern obtained on subjecting crude microsomal material derived from an acid-treated homogenate to bouyant density centrifugation indicated no tendency of the FSR fractions to reduced bouyant density.

The effect of a short period of exposure to acid conditions (pH 5,4 for 10 min at 37^oC) of minced muscle of slowly-glycolysing muscle on calcium uptake, calcium-stimulated ATPase activity and the bouyant density sedimentation behavior of FSR were examined.

The effects of acid treatment on Ca-Binding and Ca-Uptake (in the presence of oxalate) are shown in table 16 . Ca-Binding in all three fractions isolated by discontinuous sucrose gradient centrifugation was too small to be measurable after acid treatment and Ca-Uptake was diminished in all fractions (5-50% of control values). It should be mentioned that exposure of muscle homogenate to acid conditions for one half of the time (pH 5,5 for 5 min at 37^oC) elicited minimal change in the calcium-accumulating ability of FSR (results not shown). There thus appears to be a relatively long induction period in the process, after which the rate of change is very great.

(a)

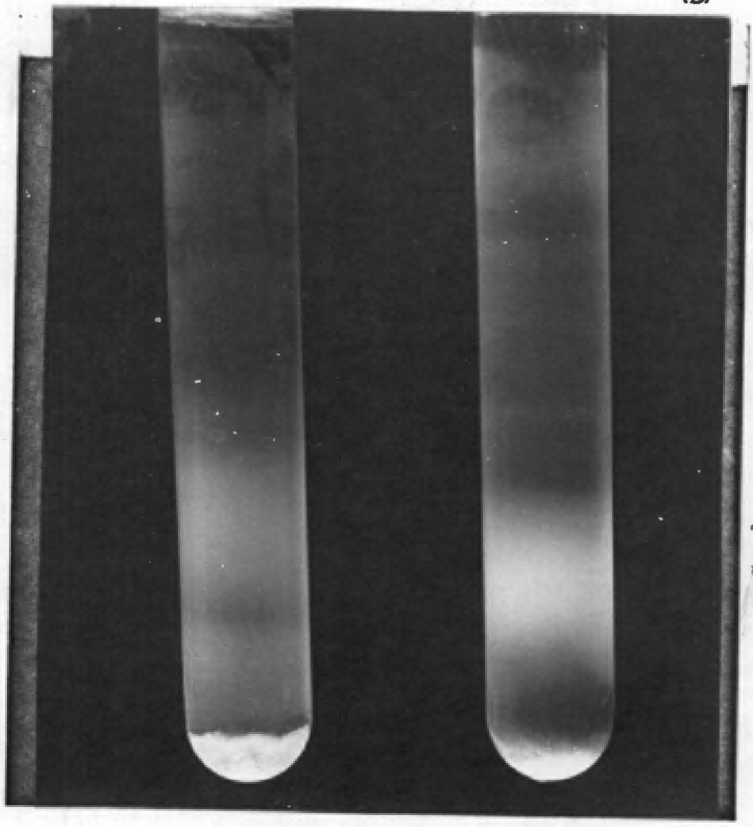


Control Acid-treated

Fig. 30 The sedimentation behavior of microsomal fraction isolated from a homogenate of slowly-glycolysing muscle following acid-treatment.

The homogenate was treated with lactic acid (pH 5,5 for 10 min at 37°C) and the microsomal fraction subsequently isolated by differential centrifugation. A suspension of microsomal material was layered on a discontinuous sucrose gradient (a) of 35, 40, 45% (w/v) sucrose and a continuous sucrose gradient (b). The discontinuous gradient was centrifuged at 64 700 xg for 2 hours and the continuous gradient for 16 hours at 60 000 xg.

(b)



Control Acid-treated

Fig. 30 cont.

Table 16 Calcium accumulation and ATPase activity of vesicles derived from slowly-glycolysing muscle and from slowly-glycolysing muscle following acid treatment.

Normal muscle (50 g) was minced and suspended in 0,1M KCl, 5 mM histidine, pH 7,20 (50 ml). The pH was lowered to pH 5,40 by the addition of a few drops of 90% L-(+)-Lactic acid and the mixture was maintained at 37°C for 10 min. It was then cooled to approximately 7°C by the addition of ice-cold 0,1M KCl, 5 mM histidine, pH 7,2 (150 ml) and the pH adjusted to 7,20. The subsequent isolation of the FSR was as in fig. 10 . Control muscle (50 g) was minced and suspended in 0,1M KCl, 5 mM histidine, pH 7,20 (200 ml) and the FSR isolated in parallel with the acid-treated preparation. The assays were performed as in Table 4. The results represent the mean of duplicate analyses of one experiment.

Fraction	Ca-Binding (nmoles Ca^{2+}. mg protein^{-1})	Ca-Uptake (nmoles Ca^{2+}/ min. mg protein^{-1})	Ca^{2+}-ATPase Activity (µmoles Pi/min. mg protein^{-1})
N_3	13,5	192	2,20
N_2	29,3	498	2,76
N_1	7,3	170	2,32
N_3 (acid treated)	$<$3,0	11,9	1,82
N_2 (acid treated)	$<$3,0	37,3	3,22
N_1 (acid treated)	$<$3,0	43,2	3,00
pellet (acid treated)	6,8	32,2	1,85

The Ca^{2+}-dependent ATPase activity of fractions derived from acid-treated muscle appears to be uninfluenced by acid treatment. In fact, the measured activity was slightly enhanced in all three fractions (table 16).

The effect of acid treatment on bouyant-density sedimentation behavior of FSR fractions on a discontinuous gradient is depicted in fig 30 . There was no tendency towards formations of less dense fractions as was observed in experiments on FSR derived from PSE muscle (fig 10). Some material after acid treatment did, however, have a bouyant density greater than 1,184 g/ml and sedimented as a precipitate at the bottom of the tube (this is not evident in the photograph, fig 30).

The sedimented material had appreciable activity in binding of calcium and in calcium uptake (table 16).

4.0 DISCUSSION.

4.0 DISCUSSION.

Calcium transport through the reticular membrane of skeletal muscle is mediated by a membrane-bound enzyme system with an absolute dependence on lipids for activity. In consequence two aspects of the transport system have been investigated (i) functional parameters of calcium transport in purified FSR and (ii) the fatty acid composition of the lipids of skeletal muscle and of purified membranes of fragmented sarcoplasmic reticulum (FSR). The discussion will be concerned with defining the nature of the defect in the calcium concentrating ability ; whether this may be related to changes in lipid composition and, hence, to the environment of the translocating system, and the use of temperature as a probe of FSR membrane function and integrity.

The particular model chosen, i.e. pale, soft exudative change (PSE) in porcine muscle was convenient and useful for studying conditions that result in the continued activation of glycogenolysis. The accelerated glycogenolysis associated with the PSE syndrome, as well as the related disorder, malignant hyperthermia, could have relevance to the physiologically increased glycogenolysis occurring during severe muscular exertion. The pH of the sarcoplasm during such exercise can also be expected to reach low levels although it will rapidly rise again after cessation of muscular activity, since lactic acid is removed into the circulatory system. Experimental conditions in this study were chosen so that a comparison could be made between skeletal muscle samples having widely differing rates of glycolysis. The samples selected for analysis evinced unequivocal gross pale, soft, exudative change concomittant with depleted glycogen content and elevated lactate levels. The control and PSE material, therefore, represent the extremes of slow and rapid rates of glycogenolysis. This consideration is of importance in evaluating the results, since, under these conditions, failure to demonstrate biochemical differences between slowly- and rapidly-glycolysing muscle would be unequivocally significant. Furthermore sampling took place within 10–15 min of death: hence, any differences between the two muscle types were either present immediately prior to death or else arose after death. This *post-mortem* interim was made up of 10–15 min during which time the muscle was in the carcass and 45 min during which time the muscle samples were in transit on ice.

The incidence of significant overall PSE change in pigs at the abattoirs was of the order of 20% (Berman *et al.*, 1972). The procedures routinely adopted for slaughter, ie stunning, immersion in a hot water-bath and firing of the carcasses are widespread practices and the incidence of PSE pork is comparable to that reported from other centres (Briskey, 1964).

Isolation and purification of FSR – Both discontinuous (Greaser *et al.*, 1969d; Seraydarian and Mommaerts, 1965; Masoro and Yu, 1971) and continuous (Hasselbach and Makinose, 1963; Boland *et al.*, 1974) sucrose gradients have been employed to obtain an enriched preparation of FSR. Purest fractions are separated as material banding in the density range 1,150–1,180 g/ml. The discontinuous gradient used in this study separated the crude microsomal fraction derived from slowly-glycolysing muscle into three bands and it was shown that the middle band ($1{,}158 < \rho < 1{,}184$ g/ml) possessed the highest specific activity of calcium accumulation and ATPase activity. The other two bands, however, also exhibited considerable activity.

It is possible that the ATPase activities of the upper and lower bands are from the same population of vesicles as the enriched middle band but became separated because of the arbitrary choice of sucrose concentrations. Separation of the microsomal fraction on a continuous sucrose gradient confirms that a disperse population of vesicles sedimenting in the central region of the gradient ($1,180 < \rho < 1,130$ g/ml) was split up on the discontinuous gradient with the result that some of these vesicles came to rest at the 40–45% sucrose interface and on the surface of the 35% sucrose layer. Analysis of the fractions eluted from the continuous gradient for ATPase activify (fig 15) indicated that all the microsomal material was active except for that occupying a narrow band in the less dense part of the gradient ($\rho = 1,100$ g/ml) and soluble proteins which remain at the top of the gradient during centrifugation. Consequently ATPase activity found in the lightest band of the discontinuous gradient (N_3) belongs entirely to fragments of the disperse band evident in the continuous gradient.

It can be concluded, therefore, that of the three fractions separated on discontinuous gradients only the middle fraction, N_2, consisted of a relatively homogenous population of vesicles. The dense band, N_1, consisted of aggregated material plus disperse fragments, both of which exhibited ATPase activity and the least dense band, N_3, consisted of active, disperse fragments plus lipo-protein aggregates and soluble proteins which were unable to hydrolyse ATP.

The distinct populations present in the microsomal fraction may represent different parts of the sarcoplasmic reticulum with specific lipid to protein ratios or may be composed of other subcellular membranes or at least contaminated by them.

Little mitochondrial contamination, estimated by the enzyme markers, cytochrome oxidase and succinate oxidase, was apparent in any of the purified fractions, which agrees with results obtained with purified rabbit FSR using a similar purification scheme (Seraydarian and Mommaerts, 1965). The presence of such mitochondrial contamination cannot be expected to alter the results significantly as Weber et al. (1966) have shown that under conditions usually used for calcium accumulation into FSR (in particular, calcium concentration approx. 1 μM) mitochondria do not take up measurable quantities of Ca^{2+} and Hasselbach (1961) observed that mitochondria do not precipitate calcium oxalate.

The plasma membrane and its continuation into the interior of the cell (termed the T-system) exhibits Ca^{2+}-stimulated ATPase activity and isolated fragments form vesicles that can accumulate calcium (Severson et al., 1972; Vasington and Murphy, 1962). However the extent of accumulation of calcium by sarcolemmal vesicles is far less than that by FSR. Severson et al. (1972) observed that the plasma membrane bound approximately 6 nmoles Ca^{2+} per mg of protein in the presence of 10^{-4}M calcium, which is 100 times the concentration of calcium required for binding to FSR.

Actomyosin fibres usually contaminate crude microsomal fractions from skeletal muscle (Greaser et al., 1969d). The fibrils tend to settle in the denser regions of a gradient and would contaminate the material in the 40-45% sucrose interface, fraction N_1. Extraction into 0,6M KCl has been shown to be effective in minimising actomyosin contamination (Greaser et al., 1969d; Uchida et al., 1965) and was routinely included in the isolation of FSR in this study.

Separation of microsomal material from PSE muscle by bouyant-density centrifugation in the discontinuous gradient employed here resulted in the consistent absence of visible and pelletable material at the 40-45% sucrose interface. There were two possible explanations for this; either the material was absent in this PSE microsomal fraction or was included in one or both of the other bands. A number of observations confirmed that the material, which from normal muscle formed a zone at the 40-45% sucrose interface, when derived from PSE muscle exhibited a lower bouyant density and moved either to the 35-40% sucrose interface (P_2) or on to the surface of the gradient (P_3). The material which appeared at the 40-45% sucrose interface in a normal gradient was distinct because of its aggregated nature. Separation of the PSE microsomal fraction on a continuous gradient clearly demonstrated an aggregated band lodged within the broad, central, disperse band (fig 15). In addition, the total amount of protein in the microsomal fraction from normal and from PSE muscle was approximately equal.

It was of interest to establish whether all the particles present in the microsomal fraction from PSE muscle in particular the material with the higher specific functional activities had a lower bouyant density when compared to comparable vesicles from normal muscle. Examination of the continuous gradients in fig 15 and those in other experiments lead to the conclusion that at least some of the vesicles present in the broad disperse band (which has the highest specific ATPase and calcium-accumulating ability) tended to form bands in regions of lower bouyant density. Occasionally, on discontinuous gradients, none of the microsomal material from PSE muscle entered even the 35% sucrose layer and in these instances it would seem that all of the most active material had a lowered bouyant density. Furthermore, it is clear that the different types of material present in the gradients were affected to a different extent. Thus the aggregated material from PSE muscle never separated completely from the disperse band and, therefore, must have lost bouyant density to a relatively greater extent than the material with which it equilibrated. The differential change of density within the crude PSE microsomal fraction emphasises its heterogeneity and maybe different locality within the muscle cell. Nevertheless, despite this variability all samples of FSR from PSE muscle had a lower bouyant-density than had normal FSR, which was reflected in their increased phospholipid/protein and cholesterol/protein ratios (see below).

The vesicles seen in electron photomicrographs are of similar size and shape to those found by Greaser et al, (1969 d) also working with pig FSR The two types of vesicles identified, namely the hollow or electron-transparent and the opaque or electron-dense varieties could be artefacts of the isolation procedure. The different proportion of each type in each isopycnic band suggests, however, that ab initio there were structural differences in the sarcoplasmic reticular fragments from which the vesicles arise.

Certain differences between the physical properties of the FSR fractions from normal and from PSE muscle were observed, apparently due to altered surface properties of the membrane. In particular, PSE membranes formed a more compact pellet after centrifugation in that this pellet had a greater tendency to adhere to the walls of the centrifuge tube. Also the reticular membranes from normal muscle were more difficult to dissolve in sodium deoxycholeate (used in protein determinations) and sodium dodecyl sulphate (used in acrylamide gel electrophoresis) than those from PSE muscle.

Calcium transport - FSR isolated from PSE muscle at 45-50 min *post-mortem* is able to bind calcium in the presence of ATP but its capacity is much less than that from normal muscle. The rate and the capacity of Ca-Uptake, in the presence of oxalate, was diminished by approximately an equal extent (by approx. 75% of normal FSR). The fact that PSE vesicles do exhibit enhanced capacity in the presence of oxalate indicates that the vesicles are able to increase the intravesicular concentration of calcium above the solubility product of calcium oxalate (approx. 1 mM). Both fractions from PSE muscle, P_2 and P_3, were equally affected and their calcium uptake per mg FSR protein was less than any of the fractions from normal muscle, indicating that the cause of the impaired activity was not due to the different distribution of microsomal material on the PSE gradient.

Assay of Mg^{2+}, Ca^{2+}-dependent ATPase activity by the pH stat method did not include oxalate in the medium and is, therefore, comparable to the conditions under which Ca-Binding was measured. The linear portion of the titration curve, occuring after 2-8 min, when the ATPase activity was measured, represents steady state conditions since ATP dependent Ca-Binding is maximal by 30 sec at 37°C. The ATPase activity measured at 37°C was not markedly altered in PSE FSR as compared to normal (fig 14). The activity in P_2 was about 75% of that in N_2 whilst that in P_3 was 5% greater than in N_3. The formation of phosphoprotein, on intermediate in ATP hydrolysis, (Martonosi, 1969) was similarly relatively unaffected by the PSE change.

The parameters Ca-Binding and Ca-Uptake are a measure of active transport through the FSR membrane (Inesi, 1972). Normally the processes of calcium transport and ATP hydrolysis are tightly coupled such that two calcium ions are translocated through the membrane for every molecule of ATP hydrolysed (Martonosi and Ferretos, 1964a). Since Ca-Binding and Ca-Uptake have been affected to a greater extent than Mg^{2+}, Ca^{2+}-dependent ATPase activity in PSE FSR, calcium translocation in these membranes can be said to be dissociated or uncoupled from ATP hydrolysis. The data tabulated in Table 4 for Ca-Binding and Ca-Uptake which indicate approximately 75% decreased activity in PSE FSR represent mean values for 7 preparations. In some preparations (two experiments) Ca-Binding and Ca-Uptake were barely detectable whilst ATPase activity was marginally lowered. These represent examples of virtually complete dissociation of the two parameters of translocation and ATP hydrolysis.

Other agents and procedures are known partially to uncouple ATPase and Ca-transport e.g. ageing (Sreter and Gergely, 1964), sonication (Sreter and Gergely, 1964), treatment with organic solvents (Inesi et al., 1967), and tryptic digestion (Stewart and MacLennan, 1974; Inesi and Scales, 1974). All of these processes, apart from tryptic digestion, appear to involve an increase in membrane permeability, with inability to maintain a concentration gradient across the membrane and consequently a decreased accumulative capacity. Sonication and organic solvents have a profound effect on membrane structure and integrity and the raised permeability gives rise to an increased steady-state ATPase activity which is unlike the situation found in PSE membranes. In spite of this, increased permeability of the PSE FSR membrane to Ca^{2+} has been considered as a possible cause for the lower maximal levels of Ca^{2+} in PSE FSR (see below).

The total quantity of calcium accumulated by FSR vesicles can be related to the rate of accumulation, the concentration gradient and the permeability of the membrane as follows

$$\Delta acc = \Delta rate \cdot P \Delta c.$$

Where Δacc = quantity of calcium accumulated, $\Delta rate$ = rate of calcium accumulation, P = permeability factor and Δc = the concentration gradient across the membrane (Inesi, 1972). Permeability depends on the composition and structure of the membrane and can be altered by such factors as temperature, detergents, organic solvents and by changing the fatty acid composition of the membrane. An estimate of permeability to calcium in the present study was obtained from the rate of efflux of Ca^{2+} from vesicles passively loaded by prolonged incubation in the presence of $^{45}CaCl_2$ and from actively-loaded vesicles, in the presence of ATP and oxalate, after inhibition of further uptake by the addition of EDTA. Both types of experiment failed to demonstrate increased passive efflux and hence augmented permeability of PSE membranes. Initial efflux rates from normal vesicles (5-25 nmoles/min. mg of protein^{-1}) was 40-100 times slower than the maximal rate of Ca-Uptake (approx. 500 nmoles/min. mg of protein^{-1}). Inesi and Scales (1974) calculated ratios of 50-100 times slower for rabbit FSR. Minor changes in efflux rate and permeability can therefore have little effect on the steady-state levels of Ca^{2+} accumulation in FSR (see also Inesi et al., 1973).

The specific rate of accumulation of calcium by PSE vesicles was diminished. This could be due to one or more of a number of factors, namely, protein contamination of PSE FSR fraction, a smaller proportion of active vesicles, a smaller proportion of ATPase molecules in each vesicle or a malfunctioning of the translocating system of PSE FSR. The diminished rate of calcium accumulation was not likely to be mainly due to protein contamination since all bands from normal microsomes showed a greater ability to accumulate calcium than did any of the PSE bands. There may have been a smaller proportion of active vesicles in PSE preparations. Greaser et al. (1969d) has demonstrated by means of calcium oxalate loading and electron microscopy that even in purified pig muscle FSR as much as 80% of the vesicles can be inactive. They were not able to determine whether this was due to localization of membranes capable of active transport in one part only of the intact SR in vivo or was due to disruption and inactivation during isolation and purification procedures. It is possible that PSE SR is more fragile or unstable and unable to withstand homogenisation as well as normal SR or has a smaller proportion of active translocating membranes in vivo. The procedure of calcium oxalate loading and visualization by electron microscopy, if applied to PSE vesicles might help to decide whether the situation in PSE FSR is due to a decreased number of active vesicles or due to a partial inactivation of each active vesicle. However even if such differentiation were possible the nature of the uncoupling of ATPase activity and calcium accumulation would still require an explanation. Boland et al. (1974) working with FSR of chicken skeletal muscle also found that only 20% of the vesicles exhibited intravesicular precipitates of calcium oxalate and that the vesicles without precipitates, when separated from the others, hydrolysed ATP at a normal rate. They suggested that these vesicles were 'leaky'.

There is the further possibility that FSR from PSE muscle did not contain as many Ca^{2+}-pump sites per vesicle as did normal FSR. The ATPase protein is considered to consist of a hydrophobic globule buried in the membrane and a "tail" which protrudes from the surface. The globule and extension are visible in electron micrographs of freeze-etched membranes and in negatively-stained preparations respectively (Tillack et al., 1974; Stewart and MacLennan, 1974). From the number of the globules and particles the density of the ATPase protein in the membrane may be calculated. An average vesicle contains about 100 ATPase sites and the number has been shown to increase with development of embryonic chicken muscle (Tillack et al., 1974). The electron micrographs of negatively-stained preparations of vesicles in this study did not allow the ready detection of the hydrophilic extentions of the ATPase protein, presumably because the magnification (120 000 x) was not high enough. The study of Greaser et al. (1969b) of vesicles from PSE and from normal muscle at 25 000 magnification does not help either. However, the similar ATPase activities and formation of the intermediate phosphoprotein suggest that the density of the ATPase sites was the same in both FSR types. Thus, it appears that diminished Ca-Binding and Ca-Uptake by FSR from PSE muscle is due to defective calcium translocation by the Ca^{2+}-stimulated ATPase "pump" system.

When analysing the nature of the translocation process, two features should be considered; firstly, the movement or transference of calcium through the intra-membranal space and, secondly, the release of calcium into the interior of the vesicle.

The rate of accumulation might be dependent on the movement of a carrier through the membrane. This movement could be the shuttling of a carrier through the membrane or else a conformational change of the carrier. In both instances, the motion would be dependent on the environment of the carrier. The presence of low-viscosity regions within FSR membranes was indicated by spin-labelling and NMR experiments (Davis and Inesi, 1972; Elect and Inesi, 1972). These regions consist mainly of the hydrophobic "tails" of the phospholipids. Metcalfe and colleagues (Warren et al., 1974) have shown that optimal conditions for ATPase activity require at least 30 phospholipid molecules per ATPase protein. Possibly the viscosity of the hydrocarbon chains of these phospholipids may determine the rate of flux through the membrane. They also showed that the ATPase protein did not have any special preference for different types of phosphatidyl cholines. This is in agreement with the results of Marai and Kuksis (1973) who failed to find any differences between the lipid composition of FSR membranes and purified ATPase complex. Thus, differences in the immediate environment of the ATPase protein could be detected by analysing the lipid composition of the whole FSR membrane. Results of such a study will be discussed below and in particular we shall consider whether any differences in the lipid composition of FSR from the two muscle types can be correlated with a malfunctioning calcium translocating system.

The other feature of the translocation that must be considered besides the movement of a carrier in the hydrophobic interior of a membrane, is that the formation of a calcium gradient requires that the binding sites on a carrier undergo cyclic changes in affinity for calcium. Inesi (1972) has suggested that such differences in affinity are related to different energy states of the ATPase calcium carrier which could be brought about by cyclical phosphorylation by ATP and dephosphorylation of the ATPase.

Martonosi (1969) proposed a carrier mechanism in which phosphorylation of the carrier by ATP on the outside surface of the FSR membrane creates a high affinity binding site for calcium. After binding of calcium the phosphorylated carrier undergoes a conformational change which results in the movement of calcium through the membrane. At the inner surface of the vesicles, the carrier is dephosphorylated with the formation of inorganic orthophosphate and Ca^{2+} is concomittantly released from the nonphosphorylated carrier which has low calcium affinity. Recent reports, however, cast some doubt on this hypothesis. It has been shown that the ATPase protein already possesses a high affinity site for calcium without the intervention of ATP (MacLennan et al., 1971; Ikemoto, 1974; Meissner et al., 1973). Also Makinose (1973) has presented evidence which shows that the formation of the high energy phosphate bond in the ATPase protein diminishes the high calcium affinity of the ATPase enzyme. Translocation could be explained by an initial binding of Ca^{2+} and ATP to the ATPase protein in a random sequence and then a simultaneous conformational change and phosphoryl transfer to the protein which eventuates in the appearance of the Ca^{2+} on the inner surface of the membrane bound to a low-affinity site. Ca^{2+} can then transfer from the ATPase to extrinsic proteins on the surface of the FSR membrane e.g. calsequestrin which possesses Ca^{2+}-binding sites of medium affinity and high capacity or,

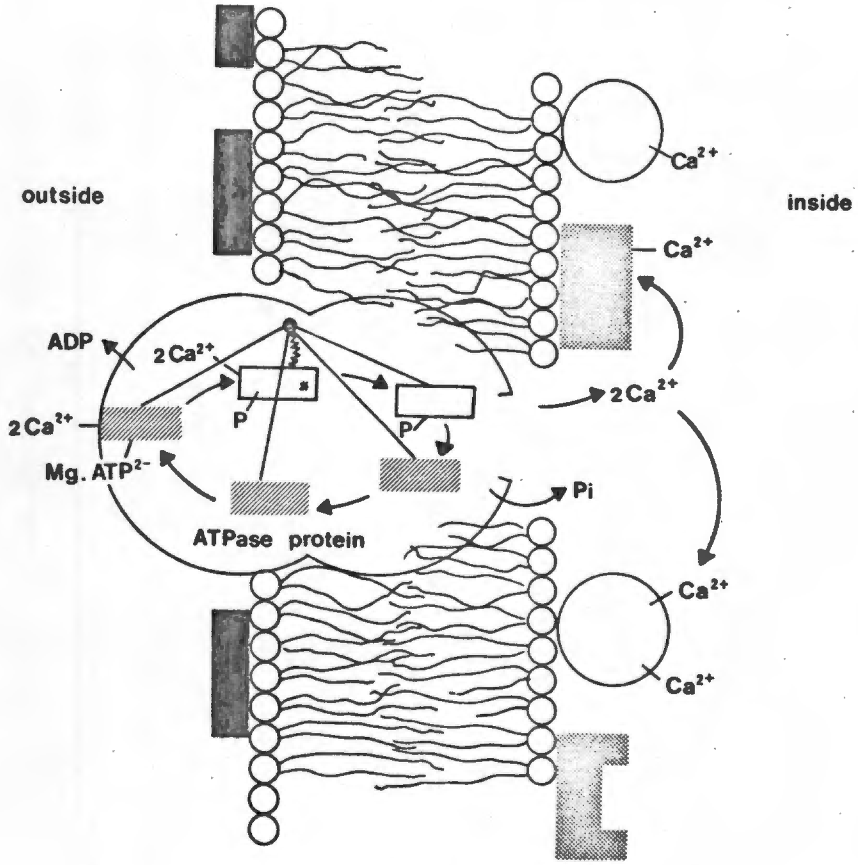


Fig. 31 Mechanism of calcium translocation through fragmented sarcoplasmic reticular membranes.

Calcium and Mg ATP^{2-} bind randomly to high-affinity binding sites (▨) on the ATPase protein. The phosphorylation of the ATPase proteins results in a conformationally altered state (*) which carries the calcium ions through the membrane and changes the high-affinity calcium binding site to one of low affinity (□), enabling the calcium ions to diffuse into the medium in the interior of the vesicle and bind to medium-affinity sites on proteins lining the membrane surface. The ATPase is dephosphorylated which restores the high-affinity of the calcium binding site and allows the return of the ATPase protein to its former conformation.

possibly, anionic sites associated with polar heads of phospholipids. (See fig 31)

Both models propose that the ATPase protein undergoes cyclical changes in its affinity for calcium with a high-affinity stage on the outside surface and a low-affinity stage on the inside. Also, there is the need for medium-affinity sites on the inside of the membrane, to keep the intravesicular calcium concentration at a concentration which permits dissociation of Ca^{2+} from the low-affinity site of the ATPase protein. A Scatchard analysis of data for passive binding of Ca^{2+} to membranes allows the resolution of binding sites with widely differing affinity. Experiments on passive binding of calcium to normal FSR membranes distinguished at least two types of binding sites (fig 23) which corresponded to medium- and low-affinity sites, as described by Chevalier and Butow (1971) for rabbit skeletal muscle FSR. These authors also identified a high-affinity site (K_D=0,4-1,3 μM) evident only if endogenous calcium was removed by a cation exchanger. This site could either be a high affinity site on the ATPase protein or one on another protein which has also been shown to possess a high affinity site, the high-affinity calcium-binding protein (Ikemoto et al. 1974). A single site of medium-affinity (K_D = 31 μM) and with a capacity of 70 nmoles Ca^{2+}/mg protein was identified for FSR from PSE muscle. Passive binding of calcium to PSE FSR by equilibration with a high concentration of calcium revealed a capacity of approximately 30 nmoles Ca^{2+}. mg protein^{-1} in the presence of 0,1 M KCl. K^+ at a high concentration would be expected to compete successfully with calcium for the low-affinity non-specific sites. This means that the PSE FSR probably also has at least two binding sites, one which is calcium specific and another which is nonspecific similar to the control membranes but each of lower capacity.

A high-affinity site for Ca^{2+} was not revealed by passive binding studies for reasons already mentioned. However, it seems unlikely that the affinity or capacity of such a site would be altered much in PSE FSR considering the similarity of activity of the ATPase and formation of the phosphoprotein intermediate to that of normal FSR. It could be argued that the calcium that stimulates ATPase activity is different from that which is translocated but Meissner (1973) observed that the ATPase protein possesses two calcium-binding sites for each phosphorylated site on the molecule and presumably these are the two positions occupied by the calcium which is translocated through the membrane with the hydrolysis of a molecule of ATP. The similarity of the kinetics of Ca-Binding and of Ca-Uptake for both FSR types also implies that the affinities of the various binding sites involved in the translocation of calcium were normal.

It might therefore be concluded that the reason for the restricted ability of PSE FSR to accumulate calcium could be an outcome of the diminished capacity of the medium-affinity, calcium-binding sites which are necessary for the translocation of calcium through the FSR membrane but not for the hydrolysis of ATP. These sites may be necessary in order to effect the transfer of Ca^{2+} from the carrier, in its low-affinity phase on the inside of the membrane, to a storage site. Alternatively, these sites could be the storage-receptors of calcium which are necessary to keep the intravesicular Ca^{2+} concentration below limiting levels. However, the fact that Ca-Uptake is diminished in parallel with Ca-Binding argues against the latter role since the anion oxalate is thought to serve such a purpose by the intravesicular precipitation of Ca-oxalate and thus would obviate the need for any medium-affinity calcium-binding sites to serve as storage sites.

Carvalho (1972) has emphasised the importance of these secondary binding sites and has presented evidence that they may reside in the phospholipids although their nature is still in doubt. Treatment of FSR membranes with phospholipases A and C increases the passive cation-binding capacity provided the products of hydrolysis are not removed (Carvalho, 1972). Their removal on albumin leads to a 50% decrease in binding capacity. A similar fate occurs if the membranal lipids are extracted with 90% acetone. Further evidence for the involvement of phospholipids in the binding of Ca^{2+} comes from reconstitution experiments in which 25% of control ATP-dependent Ca-Binding has been achieved with membranes consisting solely of ATPase protein and phospholipids (Racker, 1972). In contrast other lines of evidence suggest that extrinsic proteins of FSR may be responsible for medium- or low-affinity Ca^{2+} binding. MacLennan and Wong (1971) have isolated a calcium-sequestering protein (calsequestrin) which binds 850 nmoles Ca^{2+} per mg protein with low affinity (K_D=800 μM), and this could be the major storage site. Against this Thorley-Lawson and Green (1973) found calsequestrin to be readily extractable with EDTA, which is not thought to traverse the FSR membrane (Weber et al., 1966) and also the protein is significantly iodinated by lactoperoxidase which is also unable to permeate the membrane. Consequently calsequestrin may be located on the outside surface of the membrane where it is less likely to function as a calcium store. Carvalho (1966) was able to calculate from the pH-dependence of passive Ca^{2+} binding to FSR membranes that the pK values of the groups involved in the binding fall in the range 5-7. Few ionic species fall in this range. Seriniya and Ohki (1973) working with phospholipid monolayers, obtained pK values of 3,7 for the phosphate group and in Ca^{2+}-binding studies there was no increased Ca^{2+} binding to the monolayers in the pH range 5,5 to 7,0. In this range phospholipid ampholytes are in the zwitterionic form and possess no nett electrostatic charge owing to inter- and intramolecular assodiations eg between PO_4^{3-} and NH_3^+ and COO^- and NH_3^+ (phosphatidyl serine). Only above pH 7,0 is there a decline in NH_3^+ species leading to release of PO_4^{3-} and COO^- anions with which Ca^{2+} can associate.

The imidazole ring in proteins has a pK of 7,0 (White et al., 1954), whilst terminal-COOH groups of aspartate and glutamate have pK values of 3,65 and 4,25 respectively and these latter could provide anionic binding sites in calsequestrin which has a high proportion of these groups. Although the phosphate group and terminal-COOH groups of glutamate and aspartate fall outside the pK range of 5-7 Seimiya and Ohki (1973) obtained data that indicate ioniseable groups have a higher pK value when located on the surface of a monolayer than when present in bulk solution. This apparent shift of pK towards neutrality is thought to be due to the fact that the pH of the solvent at the surface of the phopholipid (and presumably protein) monolayer is not identical with that of the bulk solvent as a result of an attraction of counterions to the charged surface (Kegdy, 1972). Such an effect may bring the pKs of the three goups within the same range.

Separation of membranal proteins by SDS polyacrylamide gel electrophoresis did not reveal any consistent differences in the protein composition of PSE FSR as compared to normal. The protein components in porcine FSR membranes are more numerous than those from rabbit muscle. The composition of rabbit FSR was very similar to that obtained by MacLennan et al. (1972). There are many conflicting reports on the composition of rabbit FSR (Yu and Masoro, 1970; Martonosi and Halpin, 1971; Thorley-Lawson and Green, 1973; Meissner and Fleischer, 1971; Ikemoto et al.,1974; Margreth et al., 1974). The most likely reason for the increased hetergeneity of porcine FSR membranes is the presence of enzymes such as phosphofructokinase and aldolase (Margreth et al., 1974) and subunits of phosphorylase or phosphorylase b kinase

(Ostwald and MacLennan,1974). Proteolysis of the ATPase protein by proleotytic enzymes during the isolation procedure has also to be considered since Ostwald and MacLennan(1974) have shown that trypsin can progressively degrade the ATPase protein to smaller molecular weight species. The possibility that the reduced capacity for Ca^{2+} binding in PSE FSR was due to proteolytic activity of lysosomal enzymes in the low pH medium of PSE sarcoplasm was considered. However there was no consistent indication that there was any degrading of the ATPase protein to smaller fragments. Ostwald and MacLennan (1974) have shown that after tryptic digestion of rabbit FSR the proteolytic cleavage products remain associated with the membrane until extreme digestion renders the fragments below mol. wt. 10 000 at which point they dissociate. It is possible that the more severe treatment the FSR membranes are exposed to during isolation would remove proteolytic fragments of the ATPase protein and they would not appear in the FSR suspension. ATPase fragmentation could account for the decreased calcium accumulation in PSE FSR and explain the normal ATPase activity, as mild tryptic digestion has such an effect. It is not known whether such cleavage causes a reduction in the binding capacity of medium-affinity calcium binding sites and there was no evidence, even in extreme PSE muscle in which calcium translocation across the FSR membrane was almost completely lacking, of a fall in the ratio of the quantity of ATPase protein to proteins of smaller molecular size.

The possibility was therefore considered whether the lessening in the numbers of medium-affinity calcium binding sites was due to an alteration in the lipid components of the membrane. Analysis of the lipid composition of the membranes prepared from both muscle types would also allow inferences to be drawn as to the environment of the carrier molecule necessary for the translocation of Ca^{2+} and in view of the known lipid requirements for the enzyme ATPase, to establish whether any differences existed that could influence its activity.

Lipid composition of whole muscle - There is abundant evidence to show that the susceptibility to stress in pigs is genetically determined and PSE changes represents a *post-mortem* manifestation of an underlying myopathy. In Duchenne's muscle dystrophy, perhaps the best known example of myopathic change, there is also a leakage of certain enzymes such as CPK and ALD from the muscle cell to the extracellular fluid, and various lipid abnormalities have been demonstrated e.g. the fatty composition of whole muscle lipids has been shown to be altered (Takagi *et al.*, 1968). For this reason the lipid content and in particular its fatty acid composition was examined in whole muscle to establish whether any gross lipid metabolic abnormality exists in stress susceptible animals. The FSR membrane was then examined in detail to determine whether the changes found in whole muscle analysis were present in this membrane system and whether further defects could be identified.

The total lipid content of longissimus dorsi muscle (17,3 mg lipid/g wet muscle) was significantly less than that of normal muscle (34,4 mg lipid/g wet muscle). Previous investigators Allen *et al.* (1967) and Luddy *et al.* (1970) had ascertained that 4,6% of the total muscle weight of *longissimus dorsi* and 5,8% of *semimembranosus* muscles of normal porcine muscle consisted of lipid. The breed of pig was not specified in either study. Sink *et al.* (1967) measured the lipid composition of normal and PSE muscle in Poland China pigs. Their observed value of 173 mg lipid/g dry weight is virtually identical with the figure of 34,4 mg lipid/g wet weight, assuming a water content of 80% (5 x 34,4 = 172,0 mg lipid/g dry muscle). Sink *et al.* (1967) employed visual assessment as the only criterion of PSE and the initial pH values of skeletal muscle at 0 hr were only slightly different (pH 6,50 *versus* 6,29) whilst those of the present study taken at 15 min _post-mortem_ were greater (pH 6,73 _versus_ 5,69). It would appear therefore that the muscle samples used in this study represented a more extreme stage of PSE and might explain the discrepant findings.

The phospholipid content of whole muscle was not altered in PSE muscle as compared to normal muscle. Values of extracted phospholipid based upon lipid-P and assuming an average mol. wt. of 600 for phospholipids were calculated to be approximately 4,0 mg phospholipid/g wet muscle i.e. considerably less than total lipid content and comparable to values obtained by Hornstein _et al._ (1961) and by Allen _et al._ (1967). Differences observed between total lipid contents of PSE muscle and of slowly-glycolysing muscle are presumably due to a decrease in neutral lipid content of PSE muscle. The reason for this is not obvious. It is unlikely that the extraction technique (Folch _et al._ 1957) was responsible. The differences are unlikely to have arisen due to the changes occurring in the short period _post-mortem_ and probably were present already _in vivo._ Possibly the greater susceptibility to stress during life, liberation of adrenalin, augmented glycolysis and increased rate of fatty acid oxidation do not favour deposition of neutral lipid in muscle. The unaltered phospholipid content of PSE muscle suggests that the membrane content, which in fast-acting white skeletal muscle consists predominantly of sarcoplasm reticulum, is equally extensive in both muscle types.

Fatty acid analysis of whole muscle lipids showed the predominance of C16 and C18 fatty acids, in agreement with the results of others e.g. Hornstein _et al._, 1961; Allen _et al._, 1967; and Luddy _et al._, 1970. No differences in the total fatty acid composition of normal and of PSE muscle were detectable which confirms the results of Sink _et al_ (1967). The presence of dimethyl acetal derivitives of palmitaldehyde and stearaldehyde were not reported in any of the aforementioned studies. Their detection indicates the presence of alk-l-enyl diacyl glycerol (neutral plasmalogens) and alk-l-enyl phospholipids (plasmalogens).

The phospholipid fractions from the two muscle types did differ slightly but significantly in fatty acid composition. On separation of the phospholipids into the various classes, differences detectable in the total phospholipid fraction appeared in the phosphatidyl choline class; amount of linoleate was elevated and of stearate decreased in PSE muscle. In addition, lysophosphatidyl choline and sphingomyelin classes were richer in arachidonate content. Phosphatidyl choline is the most abundant phospholipid in muscle cell membranes and an alteration in its composition could have a profound effect on membrane integrity.

Each phospholipid class was observed to have a fatty acid profile characteristic of skeletal muscle. Most of the choline phosphatides of pig skeletal muscle contain palmitate, stearate, oleate and linoleate (in all 85% of total) and the predominant species can be expected to be 16:0 18:1, 16:0 18:2, 18:0 18:1, 18:0 18:2 as it has been found that usually a saturated fatty acyl chain occupies the l-position and an unsaturated side chain the 2-position of the glycerophosphatide (Kuchmak and Duggan, 1965; Marai and Kuksis, 1973; Montefoort _et al._, 1971). This would contrast with extracts from liver and kidney which have less oleate and equal amounts of palmitoyl and stearoyl linoleates (Montefoort _et al._, 1971). Porcine brain tissue on the other hand contains no linoleate and the most prevalent species is 16:0 18:1. All these tissues are distinguishable chemically from lung extracts since the latter has 16:0 16:0 as the most abundant combination. It is doubtful whether these differences merely reflect the biosynthetic capabilities of the cell type. It seems more likely that they are a reflection an intricate regulatory mechanism which controls lipid-lipid and lipid-protein interations specifically related to functions of different cells.

Phosphatidyl ethanolamine of porcine muscle was characterised by less palmitate and oleate and more arachidonate than is present in phosphatidyl choline. It was also the lipid class most lightly unsaturated. The class which contains phosphatidyl inositol and phosphatidyl serine was characterised by a large proportion of linoleate, whereas lysophosphatidyl choline and sphingomyelin had an abundance of the saturated fatty acids palmitate and stearate.

Kuchmak and Dugan, 1965, have also reported stearate to be the predominant fatty acid in the ethanolamine class of pork loin, belly, ham and ribs. The proportion of the other fatty acids was similar to the present results. Nevertheless, their findings on the composition of phosphatidyl choline differ markedly from those presented here, in that palmitate constituted 50% of the total fatty acids and relatively little stearate or linoleate was detected. The present results are more in accord with those reported by Hornstein et al.. (1961), working with unspecified port muscle and Takagi et al. (1968) analysing human muscle. The 32,4% arachidonate found by Hornstein _et al._, was, however, in excess of the 9-11% presented here. These variations are difficult to explain especially since no differences in the fatty acids of phospholipids between muscles within a carcass (Kuchmak and Dugan, 1965; Luddy _et al._, 1969) and even between species (Hornstein _et al._, 1961; Takagi _et al._, 1968) are often slight.

Coniglio *et al.*, (1954) have observed that the pool of fatty acids associated with neutral lipids has the highest metabolic turnover in skeletal muscle and have suggested that this pool supplies fatty acids to the tissue for the purpose of energy production. No differences in the fatty acid composition of the triglyceride fraction of normal and of PSE muscle were detected. The reported values are remarkably similar to those found previously in porcine skeletal muscle (Luddy *et al.*, 1970; Hornstein *et al.*, 1961). The free fatty acid fraction of lipid extracts of porcine skeletal muscle did show differences between control *versus* PSE muscle, that from PSE muscle having higher stearate (C18:0) and linoleate (C18:2) contents. The free fatty acid fraction probably contains some moeities released from phospholipids due to the action of phospholipases in the immediate *post-mortem* period or during the isolation procedure. It is therefore of interest that phosphatidyl choline of PSE muscle has relatively more linoleate than has normal muscle and that the increase in linoleate in the free fatty acid fraction may result from the action of phospholipase A which releases fatty acids occupying the 2-position of phosphatidyl choline. The fact that linoleate content of other phospholipid classes from PSE muscle was not elevated suggests that its availability was not the reason for the alteration in fatty acid composition of phosphatidyl choline.

The question as to whether the differences in fatty acid composition of the various lipid fractions of whole muscle are significant in regard to manifestations of physical and biochemical aberrations of PSE changes are best considered in conjunction with the data obtained on the lipid composition of purified membrane fractions of the sarcoplasmic reticulum.

Lipid composition of FSR membranes – the bouyant density of each band harvested from sucrose gradients could be correlated with the lipid to protein ratios of both phospholipid and cholesterol. This is particularly evident in the lightest fraction from PSE muscle, P_3, which contained significantly higher ratios of both phospholipid to protein and cholesterol to protein when compared with the analogous fraction, N_3, from normal muscle. The results further indicated that most of the vesicles present in the crude microsomal fraction from PSE muscle sedimented at a lower bouyant density level and presumably had higher phospholipid and cholesterol to protein ratios than comparable material from slowly-glycolysing muscle. Fiehn et al. (1971) have shown that the different bouyant densities of mitochondrial, FSR and plasma membranes may be related to the phospholipid content (0,31, 0,45 and 0,51 mg phospholipid/mg protein respectively) and to the neutral lipid content (eg 0,007, 0,021 and 0,156 mg cholesterol/mg protein respectively). The neutral lipid content makes the greatest contribution towards the characteristic bouyant density of each membrane type. It is therefore surprising to find that the phospholipid to protein ratio is raised in P_3. This points to the possibility that the lowering in bouyant density of PSE fragments may be due to a loss of protein associated with the membrane.

The major phospholipd of porcine FSR was phosphatidyl choline. The value obtained here (60%) is lower than those published for rabbit FSR which range from 65–73% (Drabikowski et al., 1966; Meissner and Fleischer, 1971; Martonosi, 1964; Waku et al., 1971; Marai and Kuksis, 1973). Fiehn et al., (1971) obtained a value of 58% for rat FSR and the differences between this value and the values for porcine FSR and rabbit FSR may be due to species differences.

However, Mahrla and Zachar (1974) could find no difference in a comparison of rabbit and lobster FSR. The content of phosphatidyl ethanolamine (19%) was similar to that observed by Owens et al. (1972), (18%) and by Marai and Kuksis (1973) (19%), but some of the published values for rabbit FSR are as low as 12% (Martonosi, 1964). The percentages of phosphatidyl inositol plus phosphatidyl serine, sphingomyelin and lysophosphatidyl choline vary considerably in the literature but those obtained here agree with the majority of published results. The results obtained for the FSR-enriched middle fractions N_2 and P_2 suggest that the FSR has the same phospholipid composition in both muscle types.

The most abundant phosphatide species in porcine FSR would contain choline and fatty acids, palmitate, oleate, and linoleate which is in keeping with the results of Marai and Kuksis, 1973, who found the most abundant molecular species present in rabbit FSR to be 16:0 18:1 and 16:0 18:2. The fatty acid profile of phosphatidyl choline in purified FSR is almost exactly identical with that of the same phospholipid from the total lipid extract of PSE muscle and might reflect the extensive surface area covered by the sarcoplasmic reticulum.

Phosphatidyl ethanolamine has a complex composition of fatty acids with arachidonate and other long chain hydrocarbons predominating.Marai and Kuksis (1973) found 16:0 20:4 to be the most prevalent combination followed by 16:0 18:1, 18:0 18:1 and 18:0 20:4. In contrast to intracellular membranes of most other tissues, the composition of the phosphatidyl ethanolamines of FSR is unusual in its high content of alkenyl ethers. Only the tissues of nervous tissues and heart muscle show a comparable amount (Getz et al., 1968; Klenk and Debuch, 1963). This marked difference between choline and ethanolamine phosphatides may point to differences in their biosynthesis and function.

The most abundant fatty acid in the phosphatidyl inositol (PI) plus phosphatidyl serine (PS) fraction was stearate followed by oleate and arachidonate. Marai and Kuksis (1973) found more arachidonate (24% versus 12%) and less oleate 4,8% versus 36%) than in the present study in purified rabbit FSR. Palmitate and linoleate content was also less than found in porcine FSR. The discrepancies may be related to poor resolution of PI and PS from phosphatidyl choline in the TLC system. Marai and Kuksis employed similar methodology and results on fatty acid composition of other phospholipid fractions of porcine FSR are remarkably similar to the data for rabbit FSR.

The sphingomyelin content of porcine FSR contained a large proportion of saturated fatty acids similar to sphingomyelin of other tissues e.g. heart (Karlsson, 1970). The large percentage of tetracosanoate (C24:1) has not previously been reported. The fact that this methyl tetracosanoate is eluted only after 80-90 min during isothermal GLC analysis and occurs as a rather broad peak may be contributory to its previous reported absence. The exact identity of the component C24:1, has not yet been established and is based on retention time only.

The only significant difference between the fatty acid composition of phospholipids of FSR membranes from the two muscle types was found in the phosphatidyl ethanolamine fraction which showed a decrease in arachidonate (C20:4) content from 14,5% to 11,6%.

It is pertinent at this stage to consider whether the observed differences in fatty acid composition of whole muscle lipids can be related to any structural and functional abnormality in PSE muscle membranes, inferred from the escape of proteins such as LDH and CPK from the muscle cell. Further it must be considered whether the differences found in the fatty acid composition of whole muscles extend to the FSR and whether the differences in the FSR can be related to the decreased calcium accumulating ability of PSE FSR in the light of the known lipid requirements of the ATPase protein and calcium transport.

Whole muscle analysis indicated that there was a significant difference in the fatty acid composition of phosphatidyl choline, and free fatty acid of PSE and normal muscle. The contribution of such an alteration towards a change in membrane permeability of PSE muscle membranes can be considered from the known effect of fatty acids on permeability of membranes (see 'Introduction') Factors such as degree of unsaturation of the acyl side chains of phospholipids and chain length are known to disrupt the orderly stacking of phospholipids in a membrane and alter the physical characteristics of the bilayer. The results indicated that the changes in PSE muscle occurred mainly in C18 fatty acids and the overall degree of unsaturation of the phospholipids remained unchanged. Therefore by these two criteria the stacking of phospholipids in PSE membranes can be expected to be minimally deranged. Such causal relationships are, however, difficult to establish. Duchenne's progressive muscular dystrophy which has marked degeneration of skeletal muscle fibres and a similar leakage of proteins out of the muscle cell had a similar magnitude of difference in the fatty acid profile of phosphatidyl choline when compared to control muscle as reported here . Takagi _et al_. (1968) found an increase in oleate (11,4 to 18,1%) and a decrease in linoleate (29,5 to 17,4%) in dystrophic muscle from human patients. It is not possible, however, to relate such a finding to a functional change in the membrane.

The observed alterations in lipid would assume a greater significance if they were restricted to a single membrane system such as the sarcolemma or SR. Each subcellular membrane has been proven to have a characteristic fatty acid composition and any such differential change could have operated at the synthetic level (Fiehn et al., 1971; Khandwala and Kasper, 1971). The SR extends throughout the muscle cell and probaby represents, in white muscle, the most extensive surface area of membrane. Consequently any substantial change in the fatty acid composition of phospholipids of FSR should have been reflected in the whole muscle analysis.

The results of the fatty acid analysis of FSR membranes indicate no differences in the fatty acid composition of phosphatidyl choline as found in the whole lipid extract. This is surprising and could mean that a gross change in the fatty acid composition of this phospholipid may be present in another subcellular membrane in PSE muscle.

The only difference in fatty acid composition of the phospholipids of FSR from the two muscle types was present in phosphatidyl ethanolamine, where arachidonate was found to be marginally diminished (14,5 versus 11,6%). As yet the analysis of the specific lipid requirements of the ATPase enzyme or the translocating system has not revealed a dependence on phosphatidyl ethanolamine as has been found for other enzyme systems, eg cytochrome oxidase (Brierly and Merola, 1962) and this knowledge together with the relatively minor change in arachidonate content would argue against the finding being of any significance.

Thus it would appear that the cause of reduced calcium accumulating ability of PSE FSR cannot be reasonably ascribed to an effect of lipid on the ATPase protein or due to viscosity change in the immediate environment of the carrier.

Nor can the loss of calcium binding capacity of the medium affinity sites be due to lipid alterations although it is possible that protein could mask the calcium binding sites of the phospholipids (this has relevance to protein denaturation and is considered below).

Temperature-dependence of Calcium accumulation and ATPase activity - The temperature-dependence of calcium accumulation of porcine FSR from slowly-glycolysing muscle is similar to that of normal rabbit FSR in that the activation energy for Ca-Binding was 10,5 kcal/mole and was approximately half that for Ca-Uptake (Inesi and Watanabe, 1967; Sreter, 1969). The data for PSE FSR did not allow a meaningful figure for the activation energy for Ca-Binding to be calculated but that for Ca-Uptake was similar to the control value (19,5 *versus* 22,0 k cals/moles). Inesi *et al.* (1973) noted a discontinuity at approximately 19°C in an Arrhenius plot of initial rates of Ca^{2+} accumulation in the presence of oxalate, giving activation energies of 28 k cal/mole and 17 k cal/mole for the lower and higher temperature range respectively. The value obtained here of 22,0 k cal/mole is intermediate between the two, but examination of the Arrhenius plot reveals that the data in the temperature range - 15 to 30 °C fall on a straight line and the activation energy for the process (16, 4 k cal/mole) is very similar to that of Inesi *et al.* (1973) for the same temperature range.

An Arrhenius display of the temperature-dependence of Mg^{2+}, Ca^{2+}-dependant ATPase activity of normal FSR, in the absence of oxalate, based on determinations by the pH stat method is strikingly similar to that of rabbit FSR hydrolysing ATP under steady-state conditions (Inesi *et al.*, 1973). Thus, the rabbit FSR data revealed discontinuities in the plot at 20°C and 35°C as compared to 15–18°C and 31°C obtained for porcine FSR. Although Inesi et al., (1973) did not calculate activation energy values for the individual processes, relatively high activation energy was associated in the high (above 35°C) and in the low (below 20°C) temperature ranges, and a relatively low activation energy between these temperatures. In porcine FSR the values for the activation energies of the corresponding processes were 29,9 kcal/mole and 12,3 kcal/mole respectively.

Eletr and Inesi (1972) have deduced from the use of spin labels that a discontinuity at approximately 20°C in FSR membranes is intrinsic to the lipid moeities of the membrane and is associated with a change from a gel to liquid crystalline phase due to melting of alkyl chains. Such thermal phase transitions occur in many natural membranes e.g. mycoplasma cell membranes (McElhaney et al., 1970) mitochondrial membranes (Raison et al., 1971 a and b) and in membranes composed of polar lipid extracts (Melchior et al., 1970). The transition temperature varies as a function of fatty acid composition.
Inesi et al., (1973) and Raison et al. (1971 a and b) using FSR and mitochondrial membranes respectively have demonstrated that membrane-bound enzymes are influenced by temperature-dependent lipid phase transitions. A close relationship was observed between enzyme activity and the physical state or fluidity of the membrane, largely the result of freedom of motion of the acyl chains. The high activation energy (29,9 kcal/mole) in the lower temperature range (10–18°C) can be explained as being essential to activation of the ATPase enzyme since its reactivity is limited by the highly-ordered lipid environment which restricts translational and/or rotational freedom. After melting of the acyl chains this limitation is partially removed and less energy is required to

activate the enzyme. Recent studies by Lee et al. (1974), employing nuclear magnetic resonance spectroscopy, indicate that within the membrane, lipid molecules form dense aggregated clusters surrounded by an environment of freely-dispersed phospholipid molecules. The process of melting appears to be more complex than a phase transition from a crystalline to liquid-crystalline state and is due rather to a sudden decrease in the number of clusters at the transition temperature.

The Arrhenius plot of the data on the temperature-dependence of ATPase activity of the P_2 fraction from PSE muscle is most reasonably regarded as being curvilinear, a conclusion which precludes the assignment of a meaningful unique value for the activation energy of the processes. However, at temperatures below 30°C the curve follows that of normal FSR fairly closely and it can be reasonably inferred that the two processes of high and of low activation energy in normal FSR also occur in the fragment in P_2. The P_3 fraction clearly exhibits both processes. It is interesting to note that the discontinuity between these two processes in P_3 (19°C) is 5° higher than that in the corresponding fraction from normal muscle, N_3 (14°C). Phospholipid and cholesterol to protein ratios were significantly increased in fraction P_3 as compared to N_3. It is doubtful whether this rise can be attributed to the high cholesterol content since Oldfield and Chapman (1972) found that cholesterol causes a depression of the discontinuity temperature of natural membranes and Cobon and Haslam (1973) obtained similar results with ergosterol. Phospholipid analysis of the fractions indicated a significant enrichment with phosphatidyl choline in PSE membranes and it is possible that the nitrogen bases in the phospholipids consitute another level of structural ordering. The similarities of the fatty acid composition of fractions

P_2 and N_2 cannot be extended to include the lighter fraction P_3 and N_3 and the fatty acids of the 'extra' lipids associated with P_3 may be sufficiently different from N_3 and also from P_2 and N_2 to explain the higher temperature needed to melt the alkyl chains in the low-bouyant density PSE membranes.

The change in activation energy of Mg^{2+}, Ca^{2+}-dependent ATPase activity of FSR at 31°C is of special relevance to this study since a plot of the data from PSE FSR fails to show such a transition (fig 28). Inesi and his colleagues initially explained the rise in activation energy above 35°C as being primarily due to a permeability change of the membrane at 35°C on the basis of experiments in which they measured efflux of Ca^{2+} from loaded vesicles (Eletr and Inesi, 1972). They found that if ATP-dependent Ca^{2+} accumulation by rabbit FSR is allowed to reach a steady state at 35°C and the temperature is subsequently raised to 45°C, prompt release of Ca^{2+} occured. In this context, it may be noted that at 35°C, maximal ATP-induced Ca-Binding and Ca-Uptake in normal porcine FSR (fig 26) is rapid and is followed by Ca^{2+} efflux. At 40°C the efflux is more rapid. Later results of Inesi's group (Inesi _et al._, 1973) in which the efflux was altered without interference by inward active transport, indicated that the passive efflux rate was not fast enough to interfere with active Ca^{2+} accumulation at any temperature. However, in the presence of ADP and Pi, rates of efflux comparable with the rates of accumulation were obtained. For this reason ATP-dependent Ca^{2+} binding and uptake in this study and those reported by other workers are measured at 20-25°C when efflux rates are not significant. It is not known whether conditions of high ADP and Pi prevail during the measurement of steady-state ATPase activity above 35°C.

Another explanation of the high energy of activation of ATPase activity above 31°C could be a true uncoupling of Ca^{2+} accumulation from ATPase activity. Inesi <u>et al</u>., (1973) have shown that the transition altered the motion of a spin label attached to -SH groups of the ATPase protein as well as to lipids in the hydrophobic interior of the membrane. Thermal denaturation, however, permanently abolished the transition and this result led them to suggest that the transition involved a conformational change of the ATPase protein and that this conformational change somehow affected calcium translocation.

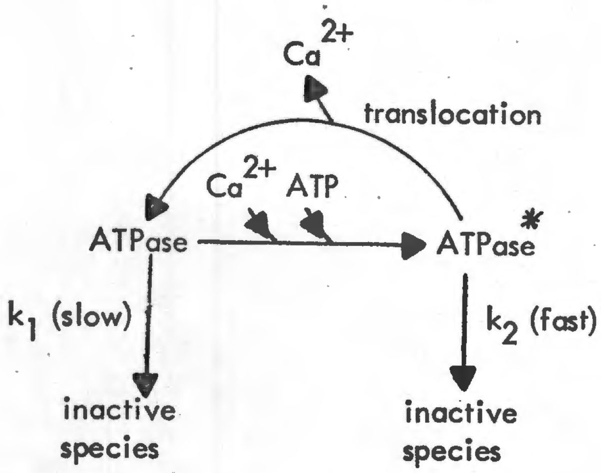
The reason for the lack of the high-activation process in PSE FSR could, therefore, be either a diminished efflux rate at higher temperatures or absence of a conformational change of the ATPase protein. There is some evidence to indicate that efflux from PSE FSR above 31°C was slower than from normal FSR. During measurements of ATP-induced Ca-Binding and Ca-Uptake in normal FSR at 35°C and 40°C, considerable efflux followed after maximal binding had occurred although adequate amounts of ATP still remained in the reaction medium. No efflux from PSE FSR was noted under similar conditions. This could be due to the fact that the amount of Ca^{2+} bound and hence the concentration gradients achieved in PSE FSR were much less than in control muscle. Another possible explanation is that efflux at high temperatures is due to a reversal of active transport, driven by the high concentration gradient of Ca^{2+} across the membrane of FSR vesicles which was established during the initial period of Ca-Binding or Ca-Uptake which was coupled to ATP synthesis in the presence of ADP and Pi. Such efflux may not be as rapid from PSE FSR, perhaps owing to low levels of accumulated calcium or to a malfunctioning of the translocation process.

If the high-activation energy process requires a conformational change of the

ATPase protein, as the work of Inesi et al. (1973) suggests, then the findings in PSE membranes of no such process lend support to the idea that a similar or identical conformational change occurs during active Ca^{2+} transport. In terms of the accepted model of Ca^{2+} translocation the cycle of binding and release of Ca^{2+} to the ATPase or pump protein is mediated by a conformational change in this protein which is coupled to ATP hydrolysis.

In the temperature range 46-50°C ATPase activity of PSE FSR was less sensitive to heat inactivation than was control porcine FSR (fig 28). This was confirmed by a decrease in the rate of thermal inactivation at 51,5°C (fig 29). Kinetic analysis of the process of thermal inactivation of ATPase activity in N_2 fragments from slowly-glycolysing muscle reveals two first order processes each with relatively rapid rate constants ($t\frac{1}{2}$=3,9 and 3,0 min). Some N_1 fragments were similarly subject to a rapid thermal inactivation process, whilst the activity of other fragments decayed more slowly with a single first order process ($t\frac{1}{2}$=10,3 min). The two fractions from PSE muscle P_2 and P_3 displayed rates of inactivation that could be described by a single first order process with $t\frac{1}{2}$=10,0 min corresponding to the slower rate constant in N_1. The light fraction N_3 displayed an inactivation process whose rate was intermediate between the slow and fast processes.

In terms of the conformation change or lack of it in PSE ATPase, suggested above, the implication is that the conformation status induced in the ATPase which is responsible for Ca^{2+} transport is relatively thermolabile whilst the uninduced state is relatively thermostable. The ATPase of PSE membranes which do not undergo this transition would therefore be thermostable.



Fraction N_1, which is not as active in Ca^{2+}-translocation as N_2, may contain a fraction of the ATPase which is unable to undergo the conformational transition. This could explain the biphasic thermal inactivation process. The intermediate decay rate for N_3 fragments may indicate another status of the ATPase protein, making the model more complex.

<u>Treatment with acid of an homogenate from slowly-glycolysing muscle</u> - A possible cause for the altered structure and function of FSR isolated from PSE muscle was the low pH prevailing in this muscle type as a result of accumulation of large amounts of lactic acid. Indeed, the results of exposure of homogenates of slowly-glycolysing muscle to low pH at 37°C, indicated that the alterations could to a large extent be explained on this basis.

However, acid-treatment did not alter the bouyant density of the microsomal fraction to the extent as occurred in PSE, i.e. the dense, aggregated fraction usually present at the 40-45% sucrose interface (N_1) did not come to rest at the 35-40% sucrose interface after treatment. This may be just a question of degree, since Greaser *et al*. (1969) have shown that muscle from stress-resistent Chester-White pigs, sampled at 24 hours *post-mortem*, contained no microsomal material banding at the 40-45% sucrose interface whereas muscle excised earlier, at time of death, contained such particles. The duration of the acid-treatment could therefore be crucial to the alterations of bouyant density.

Treatment at low pH resulted in a marked decline in Ca^{2+}-accumulating ability of the isolated FSR and could explain the observed fall off in PSE FSR, as the experiments of Greaser et al. (1969c) on acid-treated purified FSR also suggested. ATPase activity was, however, unaffected by such treatment (slightly increased if anything) also in agreement with the activity observed in PSE FSR. Acid-treatment is thus able to dissociate Ca^{2+}-accumulation from ATPase activity and can be used to investigate the nature of the link between ATP hydrolysis and Ca^{2+} translocation as well as to provide a model for the changes occurring in the FSR in post-mortem muscle particularly in PSE muscle.

Preliminary work on acid-treatment of rabbit FSR indicates that conditions can be chosen such that Ca-Uptake and Ca-Binding can be dissociated from total ATPase activity and that under more severe conditions Mg^{2+}, Ca^{2+}-dependent ATPase activity can be dissociated from Ca^{2+}-independent ATPase activity. Acid-treatment, therefore, promises to be a useful procedure to analyse the complex relationship between energy transduction in a membrane and the translocation of ions and metabolites across it. It has been further shown that proton-inactivation of Ca-Uptake results in the disappearance of the high-activation energy process present in the ATPase activity of normal fragments in the higher temperature range, 35-45°C (McIntosh et al., 1975).

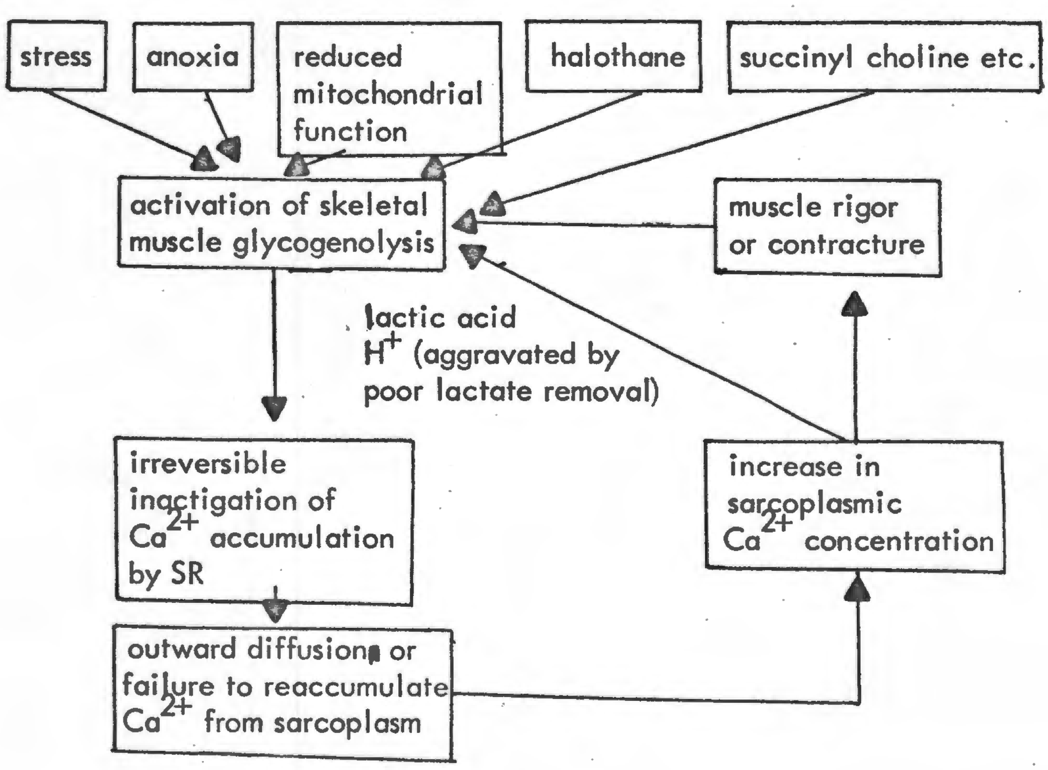
It appears therefore that many if not all of the findings may be explained in terms of proton/thermal inactivation. The characteristics of this inactivation are identical with those of protein denaturation. The relatively minor difference between the composition of the PSE membranes and normal are in keeping with this hypothesis. Denaturation of proteins can be described in terms of long-range electrostatic interactions and short-range interactions with the solvent (Tanford, 1970). The most important contribution is probably from the long-range forces where mutual repulsion between like charges in a compact conformation will favour transition to a denatured state in which charges are separated by larger distances. The denaturation process may be gradual so that initially an incompletely disordered state arises followed by transition to a random coil in which all fixed internal noncovalent interactions are disrupted. This might be relevant to the processes occurring in the FSR membrane after rapid _post-mortem_ glycogenolysis. Mild or incomplete proton-induced denaturation could result in an incompletely disordered state of the protein molecules involved in the translocation of calcium in which Ca^{2+} translocation is abolished whereas the ATPase enzyme is still capable of ATP hydrolysis. Thus a situation can be envisaged whereby partial unfolding of the ATPase molecule could prevent the conformational change necessary for Ca^{2+} translocation (Martonosi and Halpin, 1971; Stewart and MacLennan, 1974). However, in a heterogenous system such as the FSR membrane it is not possible to exclude denaturation of first one protein and then another. A loss in binding capacity of the medium-affinity sites could then be explained as being due to the unfolding of protein molecules located on the surface of the membrane which masks either binding sites on the protein itself or those on the phospholipids.

The observation that membranes from PSE muscle are more easily dissolved by SDS and sodium deoxycholate than are those from normal muscle could also be explained as the result of proton inactivation. Proteins on the surface of PSE FSR membranes may be held tightly together by noncovalent binding with a few weak sections that facilitate detergent binding. Nelson (1971) has shown that SDS miscelles do not bind to the protein surface but rather to the protein molecules as a whole. This would require some unfolding of the native proteins. Denatured proteins in which the configuration of the protein is already wholly or partially unfolded would allow easier access to the interior and hence more rapid dissolution.

The rate and severity of the pH change occurring in PSE muscle does not allow the results of the present study to be extrapolated to conditions that exist *in vivo*. Thus it cannot be inferred that any functional abnormalities exist in the PSE FSR *in vivo*. However, it is difficult to imagine how the fatty acid changes that are found consistently in whole muscle and in the individual phospholipid, cholesterol and fatty acid fractions in the FSR could have arisen as a result merely of the *post-mortem* change.

There are two affects contributing to production of hydrogen ions within a muscle (Hallund and Bendall, 1965). The first is a short term acceleration of lactate production associated with the process of recovery from muscular activity and lasts only a minute or less. This rapid glycolysis can be demonstrated in any animal whose muscle has been in tetanus. However, stress-susceptible animals, notably pigs, never recover from such stress stimulation and the rate of glycolysis continues at a rapid rate although slower than during the first acceleration. This long term effect is completely absent in rabbits. The initial pH decline in stress-susceptible pigs can be particularly severe, either as a result of hyperirritability, stress, etc., and pH values lower than those of stress-resistent pigs could ensue in the proximity of the SR membrane. Alternatively the SR in stress-susceptible animals could, as a result of a protein aberration in the FSR membrane, be particularly susceptible to pH changes. Moulds and Denborough (1974) found that the most consistent and striking feature of malignant hyperthermic muscle (an analogous state of rapid and uncontrolled glycogenolysis in live pigs and in man usually induced by anesthetic agents) is its ability to give an increased contracture when exposed to a wide variety of stimuli, including the seemingly unrelated chemical compounds, halothane, caffeine, succinyl choline, potassium chloride and the physical stimulus of temperature change. This points to an increased sensitivity of the SR membranes to many physico-chemical stimuli and could include high hydrogen ion concentrations.

On the basis of the above considerations the following simple scheme is proposed as a basis for the unexplained irreversible state of increased skeletal muscle glycogenolysis which is characteristic of both the *post-mortem* model PSE and the condition in the live animal - malignant hyperthermia.



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